SERUM FERRITIN LEVELS IN GESTATIONAL DIABETES MELLITUS

DISSERTATION

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CERTIFICATE

This is to certify that the study entitled "**SERUM FERRITIN LEVELS IN GESTATIONAL DIABETES MELLITUS**" is the bona fide work of Dr. Gopinath M, who conducted it under the guidance and supervision of Dr. Molly Jacob, MD, PhD. The work in this dissertation has not been submitted to any other university for the award of a degree**.**

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DECLARATION

I hereby declare that the investigations which form the subject matter of this study were conducted by me under the supervision of Dr. Molly Jacob, Professor of Biochemistry, Christian Medical College, Vellore.

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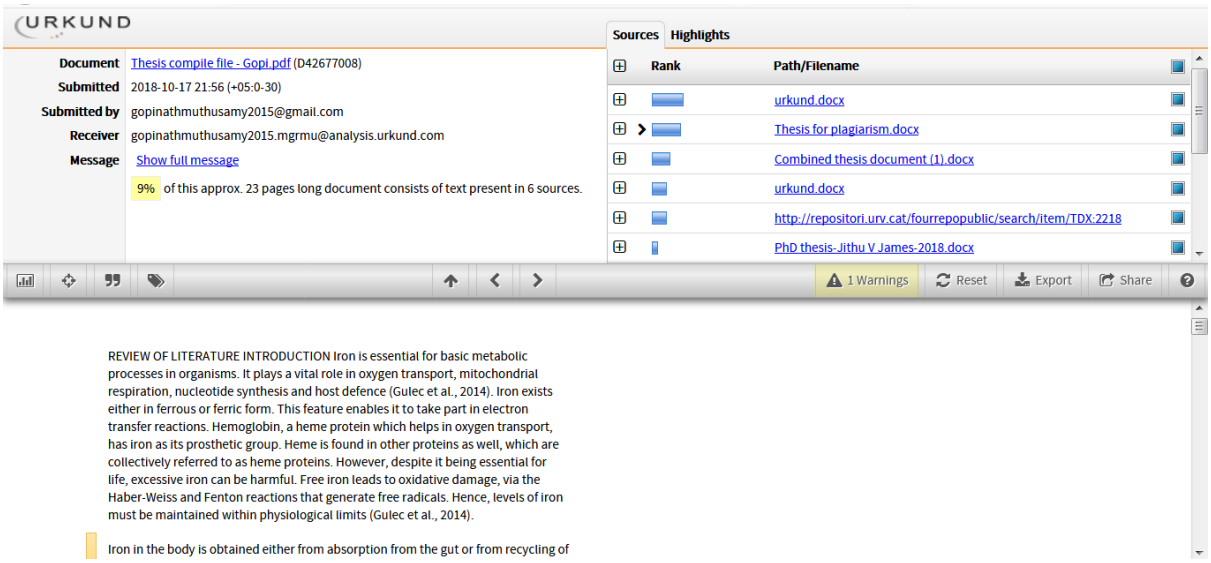
PLAGIARISM CHECK



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ABSTRACT

SERUM FERRITIN LEVELS IN GESTATIONAL DIABETES MELLITUS

BACKGROUND:

The precise mechanisms involved in etio-pathogenesis of gestational diabetes mellitus (GDM) are not well understood. Epidemiological data suggest that GDM is associated with increased iron stores in the body. Serum ferritin, often used as an indicator of body iron stores, has been shown to be increased in those with diabetes mellitus. The aim of this study was to determine whether this is so in women with GDM.

MATERIAL AND METHODS:

A case control study was carried out between January 2017 and July 2018 to recruit primigravidae, with and without GDM. Anthropometric data and a fasting blood sample were collected from each subject. The blood sample was used to estimate serum levels of ferritin, hepcidin, iron, total iron binding capacity (TIBC), percentage of transferrin saturation and C–reactive protein (CRP). Clinical data was collected from hospital records.

RESULTS:

Eighty two subjects, 23 women with and 59 women without GDM, were recruited. Primigravidae with GDM and those without GDM did not differ with regard to their haematocrit values, serum levels of iron, ferritin, transferrin saturation, TIBC, hepcidin and CRP values. Serum ferritin bore a significant positive correlation with

serum transferrin saturation, and a significant negative correlation with TIBC values. Serum hepcidin level bore significant positive correlation with serum iron, ferritin and transferrin saturation and a significant negative correlation with TIBC values. The gestational ages at delivery, birth weight of the baby, the APGAR score, and length of the babies were similar in both groups.

CONCLUSION:

There were no significant differences in levels of serum ferritin and other iron-related parameters in women with and without GDM. Hence, based on the data in this study, it does not appear that increased serum ferritin levels may be useful as a marker for development of GDM.

Key words:Gestational diabetes mellitus, iron, ferritin, hepcidin

REVIEW OF LITERATURE

INTRODUCTION

Iron is essential for basic metabolic processes in organisms. It plays a vital role in oxygen transport, mitochondrial respiration, nucleotide synthesis and host defence(Gulec et al., 2014). Iron exists either in ferrous or ferric form. This feature enables it to take part in electron transfer reactions. Hemoglobin, a heme protein which helps in oxygen transport, has iron as its prosthetic group. Heme is found in other proteins as well, which are collectively referred to as heme proteins. However, despite it being essential for life, excessive iron can be harmful. Free iron leads to oxidative damage, via the Haber-Weiss and Fenton reactions that generate free radicals. Hence, levels of iron must be maintained within physiological limits (Gulec et al., 2014).

Iron in the body is obtained either from absorption from the gut or from recycling of iron (from senescent red blood cells) in the reticuloendothelial system. The recycled iron contributes the major proportion of iron for the body's requirements. Dietary iron, which is absorbed through the small intestine, contributes much less. However, this process is highly regulated and is essential for maintaining iron homeostasis. Unlike other nutrients, there is no regulatory mechanism for the excretion of iron from the body. Thus, regulation of iron absorption is crucial (Hentze et al., 2010).

IRON METABOLISM

Absorption of iron from the diet

Iron absorption occurs in the proximal small intestine, mostly in the duodenum (Hentze et al., 2010). The amount absorbed is in the range of 1-2 mg/day, and contributes less than 10% of the body's requirement for iron. Dietary iron is found in the form of either heme or non-heme iron. The major proportion of dietary iron is non-heme iron.

A. Absorption of non-heme iron (Figure 1)

Non-heme iron in the diet is usually present in its ferric form. In the alkaline environment of the duodenum, ferric iron forms insoluble complexes (Przybyszewska and Żekanowska, 2014). Duodenal enterocytes cannot take up the ferric form of iron (Wollenberg and Rummel, 1987). In order to facilitate iron absorption, the ferric form must be converted to its ferrous form. This is accomplished by a brush border ferri-reductase, duodenal cytochrome b (McKie et al., 2000). This is a transmembrane protein that is located on the apical surface of duodenal enterocytes (McKie et al., 2001). It is a member of the cytochrome b561 family of proteins, the expression of which is regulated by iron. Its mRNA and protein levels have been shown to increase in iron-deficiency states and in presence of hypoxia (McKie et al., 2001).

The apical membrane of enterocytes has a protein called divalent metal ion transporter 1 (DMT1) that takes up the ferrous iron, which is then transported into the cytosol

(Gunshin et al., 1997). DMT1 belongs to a family of proteins known as natural resistance-associated macrophage proteins (NRAMP). DMT1 is also known as NRAMP2 or DCT1 (divalent cation transporter 1). It is a non-specific transporter that transports other divalent cations such as Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+} and Ni^{2+}, besides iron (Gunshin et al., 1997). DMT1 is also present in erythrocytes, where it plays a role in pumping iron out of the endosome during the transferrin cycle, explained below (Andrews et al., 1999).

Iron that enters the enterocyte undergoes one of two fates, depending on the body's requirements for iron. It is either stored in the cell (as ferritin, which is a storage protein for iron)(Arosio and Levi, 2010)or released into the circulation. Iron inside enterocytes is exported into the circulation, by ferroportin, which is present on the basolateral surface of enterocytes (McKie et al., 2000). It is encoded by the *SLC40A1* gene and belongs to the family of metal transport 1 proteins (Abboud and Haile, 2000; McKie et al., 2001). It is also referred to as IREG1 (iron-regulated transporter 1) or MTP1 (metal transport protein 1)(Abboud and Haile, 2000).It is the only known iron exporter identified in mammals (Abboud and Haile, 2000). It is highly expressed in macrophages, duodenal mucosal cells, hepatocytes, Kupffer cells and placental syncytiotrophoblast cells (Abboud and Haile, 2000). Ferroportin knockout mice have been shown to have iron-deficiency anemia, highlighting the importance of ferroportin in iron absorption (Donovan et al., 2005).

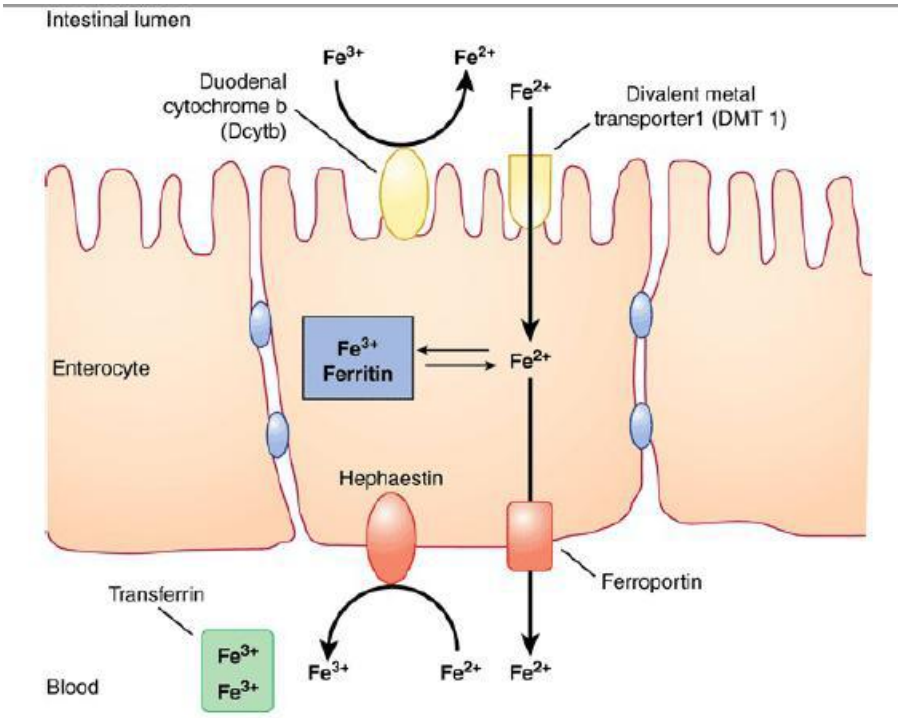
The duodenal enterocyte also has an enzyme on its basolateral surface, hephaestin, which is a ferroxidase that converts the iron into its ferric form (De Domenico et al.,

2007; Vulpe et al., 1999).It is this form that can bind transferrin, which is the protein that transports iron in blood (Schade and Caroline, 1946).

B. Absorption of heme iron

Digestive enzymes secreted in the intestine cause proteolytic degradation of heme proteins. This results in separation of heme from the protein. The heme is taken up into enterocytes by a heme transporter, present on the apical surface of the membrane of these cells (Shayeghi et al., 2005). Inside the enterocytes, hemeoxygenase (HO-1) releases ferrous iron from heme(Raffin et al., 1974). The released iron follows the same fate as that of non-heme iron.

Figure 1: Absorption of iron in the intestine



Source: Rodwell, V., Bender, D., Botham, K.M., Kennelly, P.J., and Weil, P.A. (2015). Harpers Illustrated Biochemistry 30thEdition (McGraw Hill Professional).

Recycling of iron by macrophages

A major part of iron in the body is present in the form of hemoglobin in red blood cells (RBCs). Senescent RBCs are phagocytosed by macrophages of the reticuloendothelial system, leading to the formation of phagolysosomes. Cytosolic heme oxygenase-1 (HO-1) in the macrophages degrades the heme, releasing ferrous iron in the phagolysosome(Poss and Tonegawa, 1997). This is transported out of the endosome into the cytoplasm by DMT1 (Tabuchi et al., 2000). The ferrous iron then either enters the labile iron pool, from where it is transported out of macrophage by ferroportin(Donovan et al., 2005),or it is stored in the cell as ferritin. Ceruloplasmin, a copper-containing plasma protein in circulation, converts the released ferrous iron to its ferric form, which then binds to transferrin in circulation (Harris et al., 1999).Iron released by macrophages is the major source of iron for the requirements of the body (Zhang and Enns, 2009).

Iron in circulation

Ferric iron is insoluble; hence, it is transported in blood bound to apo-transferrin (Baker et al., 1994).Transferrin is a bi-lobed glycoprotein synthesized by the liver. Apo-transferrin has 2 iron-binding sites, which reversibly bind ferric iron (Luck and Mason, 2012). Under physiological conditions, about 30-40% of the binding sites on

transferrin are occupied by iron. This represents transferrin saturation, which is a reflection of the iron status of the body, and hence a clinically useful marker(Luck and Mason, 2012). Under physiological conditions, all the iron in circulation is transferrin-bound. When there is an iron overload, the excess amount of circulating iron exceeds the binding capacity of transferrin (Barisani et al., 1995). This results in iron in circulation that is not bound to transferrin. This unbound iron complexes with citrate and albumin in circulation, and is referred to as non-transferrin bound iron (NTBI) (Arezes et al., 2013).

Uptake of iron by tissue

Transferrin, with its bound iron (holo-transferrin), in circulation is taken up into cells by receptor-mediated endocytosis. Cells have transferrin receptors on their surface. Two types of transferrin receptors are known, transferrin receptors 1 and 2 (TfR1 and TfR2) (Kawabata et al., 1999). TfR1 is present in all cells, while TfR2 is present in hepatocytes and small intestinal crypt cells. The function of TfR1 is to facilitate uptake of transferrin into cells, whileTfR2 functions as an iron sensor. Holo-transferrin binds to its receptor and is endocytosed. The early endosome, thus formed, is acidified by Na^{+}-H^{+}-ATPase, which pumps H^{+} into the endosome. This lowering of pH causes a conformational change in the transferrin, resulting in dissociation of ferric iron from transferrin (Dautry-Varsat et al., 1983). The ferric iron is converted to ferrous form by an endosomalreductase - six transmembrane epithelial antigen of prostate 3 (STEAP3) (Ohgami et al., 2005). The ferrous iron is then transported out of

the endosome into the cytosol by DMT1(Fleming et al., 1999). Thus, DMT 1 is not only involved in the absorption of ferrous iron from the intestinal lumen, but it is also essential for the transport of ferrous iron from the endosome into the cytosol after erythrophagocytosis(Hentze et al., 2010). The iron is then either utilised by the cell or stored in ferritin. The apo-transferrin and the transferrin receptor are recycled back to the cell surface, where the apo-transferrin dissociates from its receptor and re-enters the circulation (Dautry-Varsat et al., 1983). In physiological states, the uptake of transferrin-bound iron by TfR1-mediated endocytosis is the major route of iron uptake in cells (Hentze et al., 2010). The cytoplasmic labile iron pool (LIP) is used by erythroid cells for synthesis of heme or iron-sulphur clusters, or stored as ferritin in the case of other cells.

In iron-overloaded states, there is an increase in circulating levels of non- transferrin bound iron (NTBI). The exact mechanism by which NTBI is formed is not clearly understood. It has been proposed that it may involve one or more ferri-reductases on the surface of cells (Ji and Kosman, 2015). NTBI is taken up into cells by ZRT/ IRT-like proteins (ZIPs), namely ZIP14 andZIP18 (Jenkitkasemwong et al., 2012; Liuzzi et al., 2006). After uptake, the iron enters the labile iron pool and follows the same fate as iron from the transferrin cycle.

Ferritin

Excess iron in the intracellular labile iron pool is sequestered in ferritin (Arosio and Levi, 2010). The ferritin molecule is a hetero-polymer of 24 subunits, which form a

hollow space in the centre. The subunits are composed of heavy (H) and light (L) chains. The ferrioxidase activity of H-ferritin enables it to take up ferrous form and store it in its ferric form, which is then attached to the glutamyl group of L-ferritin (Arosio and Levi, 2010). Each ferritin molecule can store up to ~ 4500 iron atoms in the form of non-mineralized ferric hydrate iron (Arosio and Levi, 2010). The transport of iron into ferritin is carried out by ribonucleoproteins known as poly(rC)-binding proteins 1-4 (PCBP 1-4), which act as chaperones (Lane et al., 2015). The iron in ferritin can be mobilized in times of cellular need (Saito, 2014).

Ferritin is found in circulation in small quantities. The source of this is not clear. Ferritin in blood is chiefly made up of L-chains, which are glycated and poor in iron content(Santambrogio et al., 1996). Serum ferritin levels reflect the body's iron stores (Lipschitz et al., 1974); levels increase in iron-overloaded states. However, serum ferritin is also an acute phase reactant. Its levels are raised in response to inflammation. Hence, serum ferritin is not a reliable marker of iron overload in the presence of inflammation (Lipschitz et al., 1974).

Regulation of intracellular iron homeostasis: IRE/IRP system (Figure 2)

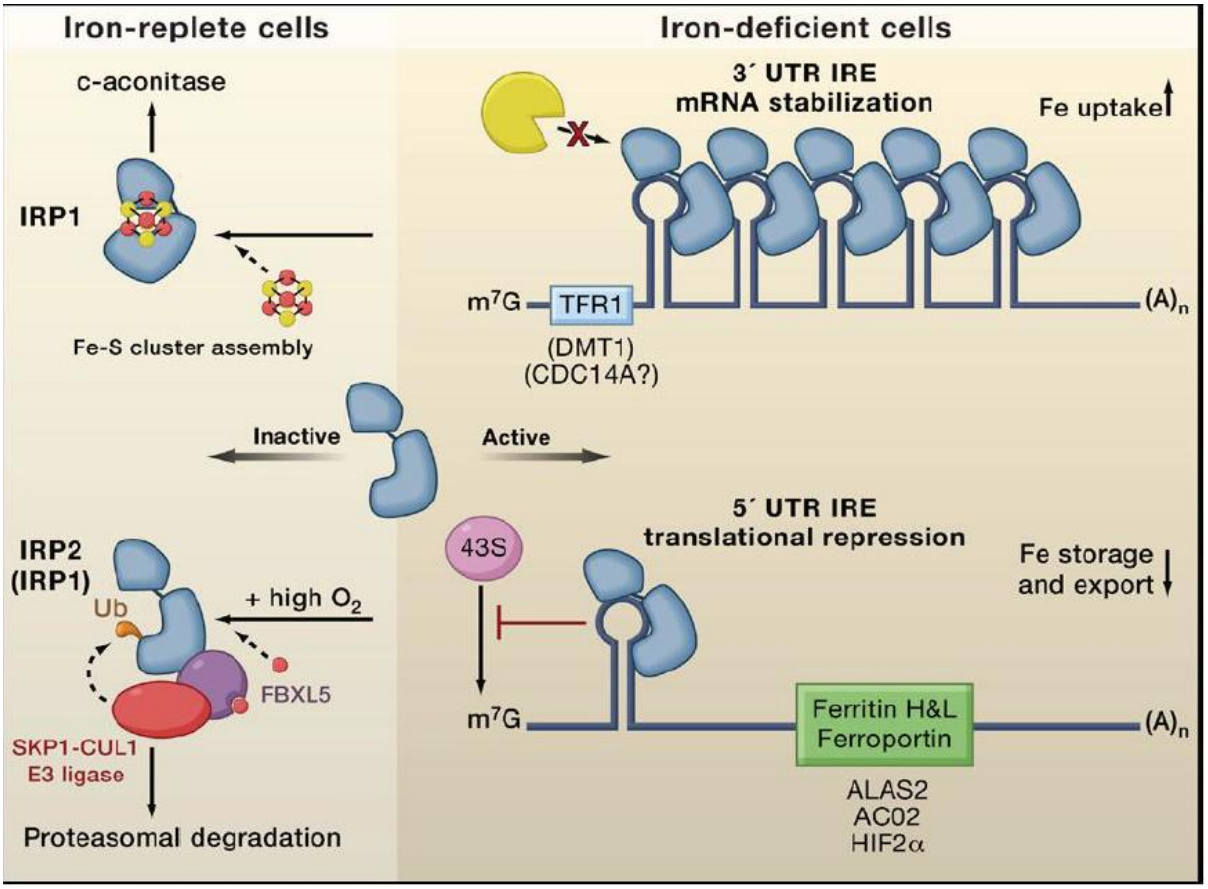
Intracellular iron homeostasis is regulated by processes that involve post-transcriptional changes and by alterations in the stability of mRNA (Hentze et al., 2010). The mRNA for iron-related proteins contains hairpin loop-like domains in the 5' or 3'untranslated region (UTR). These are known as iron-responsive elements (IRE)(Hentze et al., 2010). IREs contain binding sites for iron-regulatory proteins

(IRP). There are two types of IRPs- IRP1 and IRP2. They are orthologs, which have the same function but are regulated in different ways. IRPs are RNA-binding proteins. They interact with iron-responsive elements (IREs) in the 5' or 3' UTR regions of the target mRNA. On binding, they either stabilize or destabilize the mRNA. This outcome is dependent on the location of IRE on the mRNA (Hentze et al., 2010). If the IRE is on the 5' end, then the binding of IRP will lead to inhibition of translation. Studies have shown that ferritin (H and L chain), ferroportin, erythroid ALA synthase **(**ALAS2**)**, mitochondrial aconitase and hypoxia-inducible factor-2α **(**HIF-2α**)** have IREs in the 5' end (Anderson et al., 2009; Leibold and Munro, 1988; Rouault, 2005; Zheng et al., 1994; Pantopoulos, 2004).

If the IRE is on the 3' end, binding of IRP will result in stabilization of mRNA, thereby promoting translation. IREs on the mRNA for transferrin receptor1 (TfR1) and DMT1are at its 3' end (Mackenzie et al., 2008). Binding of IRP to the 3' end of mRNA results in increased translation of transferrin TfR1and DMT1.

Iron-regulatory proteins are induced when iron levels are low and they bind to mRNA. When iron levels are high, iron-sulphur clusters are formed, which binds to IRP and prevent their binding to IREs. The IRPs then undergo ubiquitination and proteosomal degradation (Hentze et al., 2010). When cellular iron is low, the iron-sulphur clusters are not formed and the IRPs are free to bind to the IREs. The overall effect is decreased utilization and storage of iron, with increased cellular uptake of iron (Hentze et al., 2010).

Figure 2: Regulation of intracellular iron levels



Source: Hentze, M.W., Muckenthaler, M.U., Galy, B., and Camaschella, C. (2010). Two to Tango: Regulation of Mammalian Iron Metabolism. Cell *142*, 24–38.

Systemic iron homeostasis

As mentioned earlier, there is no regulated mechanism for excretion of iron from the body. Systemic iron homeostasis is thus tightly regulated at the level of intestinal absorption. Hepcidin is the central regulator of the processes involved(Hentze et al., 2010).It is a peptide with anti-microbial properties, synthesized in the liver as a pro-hormone. A pro-hormone convertase, furin, cleaves the pro-hormone to form hepcidin, which contains 25 amino acids (Valore and Ganz, 2008). When first discovered, it was called liver-expressed anti-microbial peptide (LEAP 1) (Krause et al., 2010). It was later named hepcidin(Park et al., 2001). In circulation, it is bound to

α2-macroglobulin(Peslova et al., 2009). It either undergoes excretion via the kidneys or degradation, along with ferroportin (as described below) (Hentze et al., 2010).

The link between hepcidin and iron metabolism was initially unknown. Many studies have provided much evidence of links between the two. Hepcidin expression was found to be increased in iron-loaded mice (Pigeon et al., 2001). A complete defect in expression of hepcidin and associated tissue iron overload were observed in mice in which upstream regulatory factor (USF) had been knocked out. Severe iron-deficiency anemia was observed in transgenic mice with hepcidin over-expression (Nicolas et al., 2002).

Hepcidin has been shown to bind to ferroportin, present in enterocytes, macrophages and syncytiotrophoblastsin the placenta (Abboud and Haile, 2000; Donovan et al., 2005; McKie et al., 2000). This causes its internalization and subsequent degradation (Nemeth et al., 2004b). Low hepcidin levels have been shown to be associated with increased expression of ferroportin(Viatte et al., 2005).

Regulation of hepcidin synthesis

Hepcidin synthesis is regulated at the level of transcription, and is influenced by numerous factors such iron status, erythroid activity, hypoxia and inflammation. It is negatively regulated by low iron levels and increased erythropoiesis. The resultant decreases in hepcidinincreases iron absorption from the gut and recycling from macrophages. Positive regulators include increased iron levels and inflammation (Hentze et al., 2010). Hepcidin is, in fact, a type 2 acute phase protein (Nemeth et al.,

2003). Inflammation causes upregulation of the transcription of hepcidin, via cytokines such as IL-6. This results in decreased iron absorption and promotes sequestration of iron in macrophages (Nemeth et al., 2004).

A. Regulation by iron levels

Two main signalling pathways are involved in regulation of hepcidin synthesis by circulating iron levels.

a. Hfe-TfR2 (HFE-transferrin receptor 2) pathway

b. HJV-BMP (hemojuvelin–bone morphogenic protein) pathway

a. Hfe-TfR2 signalling

Current knowledge of the role of the Hfe-TfR2 pathway in regulation of iron homeostasis is based on observations of various mutated membrane proteins (HFE, HJV and TfR2) in hereditary hemochromatosis. Hfe and transferrin receptor-2 are transmembrane proteins in hepatocytes. Mutations of these proteins were observed in patients with hereditary hemochromatosis (Feder et al., 1996; Roetto et al., 2002). In such patients, it was found that, despite the iron overload, they were found to have low hepcidin levels. The iron overload was held to be due to increased iron absorption in the presence of low levels of hepcidin(Bridle et al., 2003; Nemeth et al., 2005; Papanikolaou et al., 2004). Nicolas et al (2002) showed that crossing $Hfe^{-/-}$ and hepcidin over-expressing transgenic mice resulted in mice that constitutively expressed hepcidin, thereby preventing iron overload, an observation that was in contrast to what is seen in $Hfe^{-/-}$ mice.

Hfe is a transmembrane protein expressed in hepatocytes (Zhang et al., 2004). Structurally, it resembles the class I major histocompatibility complex (MHC), with α1, α2 and α3 chains, associated with β2-microglobulin(Li et al., 2016). Transferrin receptor-1(TfR-1) is present in most cells and enables iron uptake, via holo-transferrin. TfR-1 has binding sites for both Hfe and holo-transferrin; these sites overlap each other. It can only bind either Hfe or holo-transferrin at a time (Feder et al., 1998), with a greater affinity for holo-transferrin. Hence, binding of holo-transferrin to TfR1 results in dissociation of Hfe from TfR-1. In keeping with this, increased concentration of holo-transferrin was found to displace Hfe from TfR1(Gao et al., 2010).TfR-2 is also a transmembrane protein; it is involved in sensing iron levels (Kawabata et al., 1999). Unlike TfR-1, TfR-2 can simultaneously bind Hfe and holo-transferrin (Chen et al., 2007).

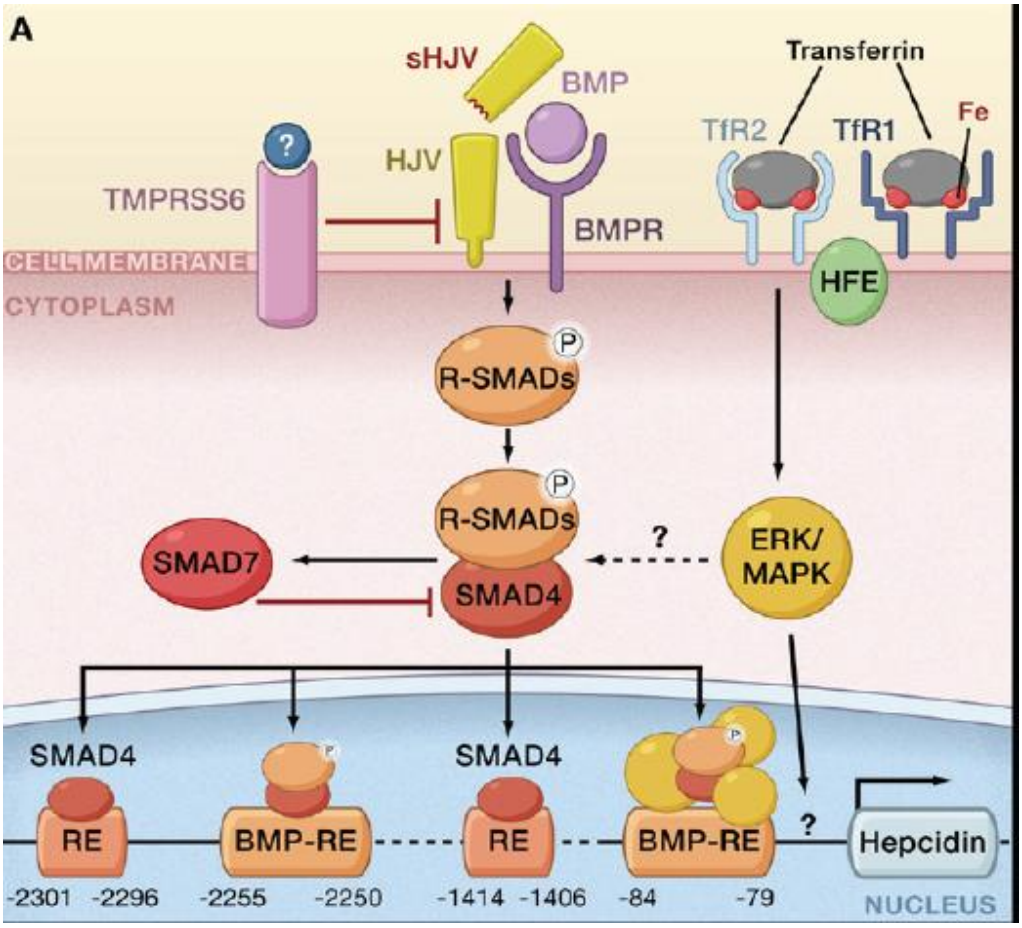
In order to study the binding affinity of TfR1 for Hfe, Schmidtt et al., (2008) engineered mouse models with TfR1 mutations. Mutations in TfR1, which produced an increased affinity for Hfe, resulted in decreased hepcidin levels and subsequent iron overload, as seen in hemochromatosis. On the other hand, mutations of TfR1 that produced decreased binding affinity for Hfe resulted in increased hepcidin levels and iron deficiency (Schmidt et al., 2008).

b. *HJV-BMP (hemojuvelin–bone morphogenic protein) pathway*

Bone morphogenic proteins (BMP) belong to the transforming growth factor β(TGF-β) family. They are involved in cell proliferation, differentiation and cell signalling. Binding of BMP to its receptor on the cell membrane occurs with the aid of a co-

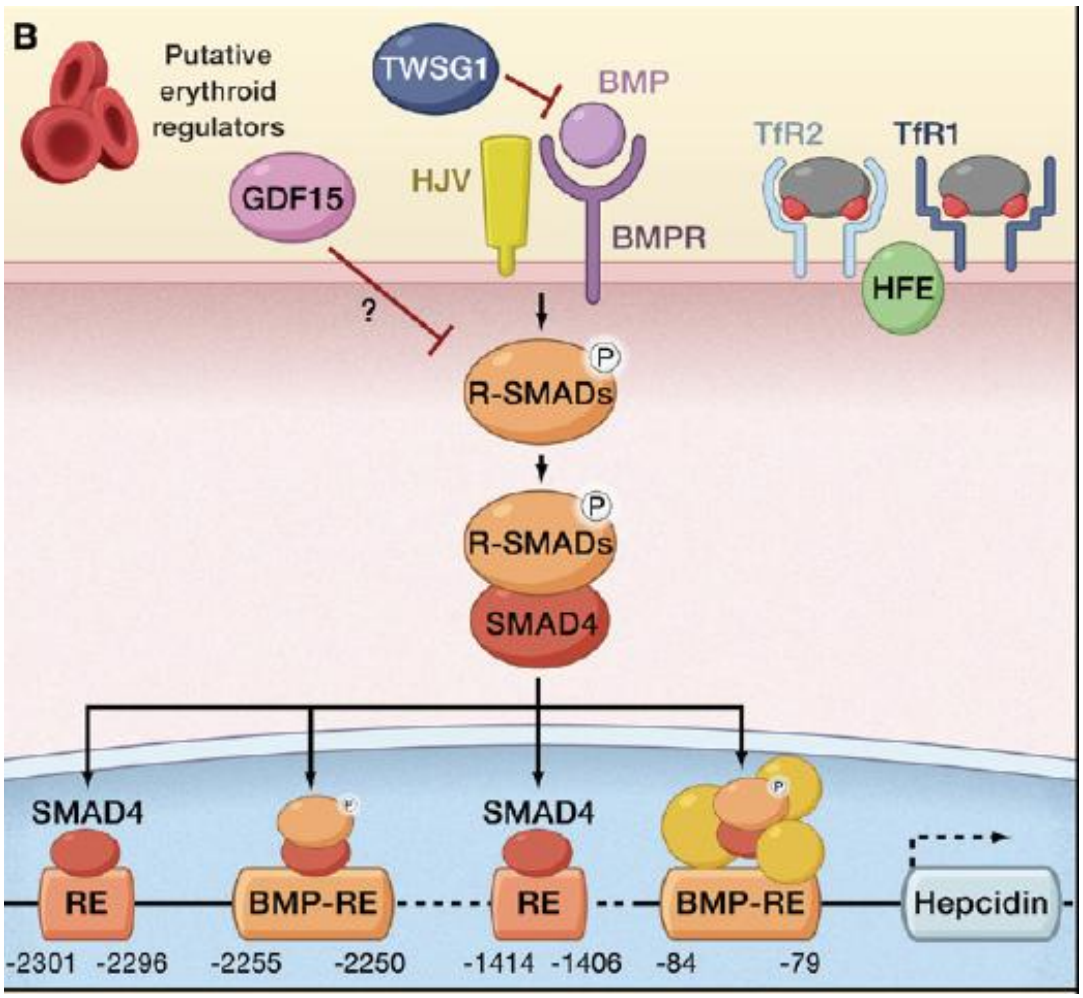
receptor hemojevulin(HJV). The binding causes dimerization of type I and II BMP receptors (serine/threonine kinase), (Xia et al., 2008). This induces phosphorylation of receptor-regulated R-SMAD. This, in turn, results in R-SMAD forming a complex with SMAD4, which induces the transcription of hepcidin by binding to its promoter (Shi and Massagué, 2003). Many types of BMP proteins have been identified. Of these, BMP-6 is the main ligand for the HJV-BMP pathway in the liver (Enns et al., 2013). Matripase-2,which is a transmembrane serine protease encoded by the *TMPRSS6*gene, is expressed in the cell membrane of hepatocytes (Velasco et al., 2002). Mutations in the *TMPRSS6* gene resulted in an iron-refractory iron-deficiency anaemia (IRIDA) (Finberg et al., 2008). Patients with this mutation also had high hepcidin levels. It has also been shown that *TMPRSS6* gene expression was increased in iron-deficiency anaemia and hypoxia (Talbot et al., 2011). Matripase-2 cleaves the membrane-bound HJV. Since HJV acts as a co- receptor for BMP, its cleavage inhibits hepcidin transcription via the BMP/SMAD pathway (Silvestri et al., 2008). Iron and BMP-6, which induce hepcidin, have beenshown to increase expression of matriptase. These findings suggest that matriptase 2 may be involved in fine-tuning of hepcidin synthesis, by preventing prolonged up- regulation of hepcidin(Meynard et al., 2011).

Figure 4: Regulation of hepcidin by systemic iron availability



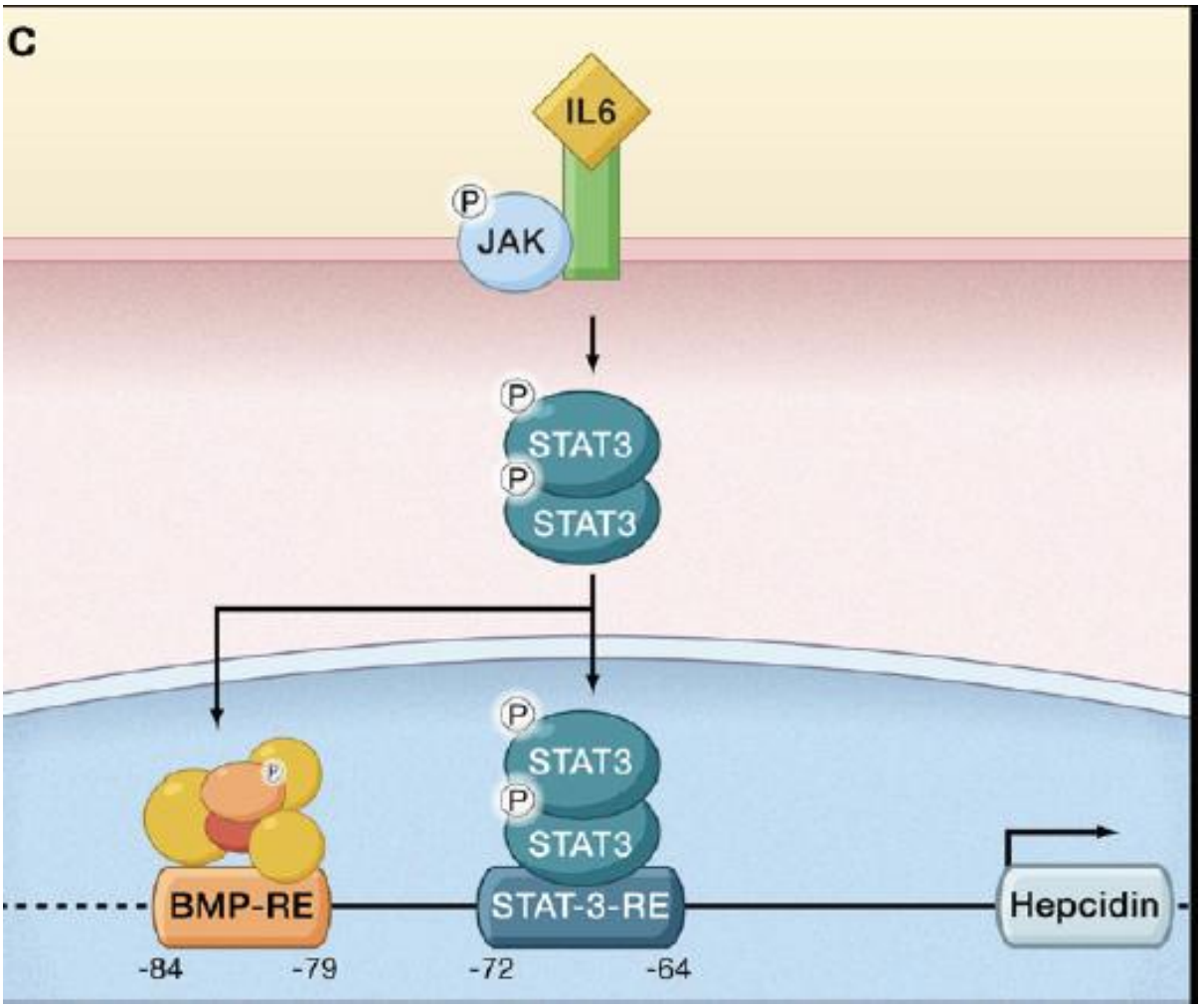
Source: Adapted from Hentze, M.W., Muckenthaler, M.U., Galy, B., and Camaschella, C. (2010). Two to Tango: Regulation of Mammalian Iron Metabolism. Cell 142, 24–38.

Figure 5: Regulation of hepcidin by erythroid signalling



Source: Adapted from Hentze, M.W., Muckenthaler, M.U., Galy, B., and Camaschella, C. (2010). Two to Tango: Regulation of Mammalian Iron Metabolism. Cell 142, 24–38.

Figure 6: Regulation of hepcidin by inflammation



Source: Adapted from Hentze, M.W., Muckenthaler, M.U., Galy, B., andCamaschella, C. (2010). Two to Tango: Regulation of Mammalian Iron Metabolism. Cell 142, 24–38.

B. Regulation by erythroid signals

Erythropoiesis requires iron. Erythropoietin, produced in the liver and kidneys, is the major hormone involved in the process. Increased erythropoietin levels are seen in anemic and hypoxic states. Studies in healthy volunteers have shown that administration of erythropoietin resulted in decreased hepcidin levels (Robach et al., 2009). Hepcidin expression was found to be suppressed in participants who underwent phlebotomy(which increased erythropoiesis)(Ashby et al., 2010). During erythropoiesis, various erythroid factors are released from the bone marrow. These include growth differentiation factor 15(GDF15), twisted gastrulation factor (TWSG1) and erythroferrone (ERFE). Increased levels of GDF15 have been reported in thalassemic patients (Tanno et al, 2007). In thalassemic mice models, TWSG1 and ERFE were found to be increased (Mirciov et al., 2017).These observations were associated with low hepcidin levels. It has been postulated that these erythroid factors act to downregulatehepcidin synthesis, by inhibiting the HJV/BMP pathway (Tanno and Miller, 2010; Kautz and Nemeth, 2014).

C. Regulation by inflammation

Hepcidin is an acute phase reactant (Nemeth et al., 2003). IL-1 and IL-6 increase the expression of hepcidin(Nemeth et al., 2004). Such inflammation-induced upregulation of hepcidin results in decreased serum iron levels, due to sequestration of iron in macrophages and also due to decreased intestinal absorption. IL-6 acts through the Janus kinase/ signal transducer and activator of transcription (JAK/STAT) pathway and increases hepcidnin transcription (Wrighting and Andrews, 2006). Cross-talk has

been shown to occur between interleukin-mediated upregulation of hepcidin(via theJAK/STAT pathway)and the BMP/HJV/SMAD pathway via SMAD4 (Wang et al., 2005).

PREGNANCY

Pregnancy, a normal physiological process, extends over a period of 280 days or 40 weeks, counting from a woman's last menstrual period to child birth. The 40-week period is divided into first, second and third trimesters, with each trimester comprising a period of 13 weeks (Hill and Pickinpaugh, 2008).

Pregnancy and iron metabolism

The body undergoes several physiological changes during pregnancy, in order to meet the needs of the growing fetus(Hill and Pickinpaugh, 2008).The body's requirement for iron is increased in pregnancy, as it is essential for development of the placenta, the growing fetus, to maintain RBC mass and to compensate for blood loss during delivery (Koenig et al., 2014).

The placenta is vital for gas exchange and for nutrient delivery to the fetus. Iron deficiency during pregnancy can result in preterm birth and low birth weight. This is due to the various pathological processes that occur as a result ofiron deficiency (Allen, 2000). Hypoxia is known to initiate the production of corticotrophin-releasing hormone (CRH), which in turn, leads to cortisol secretion by the fetus that can cause preterm delivery (Gülmezoglu et al., 1996). Iron deficiency has been reported to leads

to poor immune response, which results in, increased susceptibility to infection causing spontaneous preterm birth (Bhaskaram, 2001).

Placental transfer of iron

In maternal circulation, iron bound to transferrin (Tf) is taken up by the fetusvia TfR1, found in the syncytiotrophoblasts lining the placenta on the maternal side (Kawabata et al., 1999). On binding to its receptor in the placenta, the Tf-TfR1 complex is taken up by endocytosis and a vesicle is formed in the cytosol. Acidification of the vesicle follows. The low pH produced causes dissociation of iron from transferrin (Harris et al., 1999). The iron from the vesicle leaves via DMT1on the endosomal membrane and is used for cellular needs. If in excess of cellular requirements, iron taken up is stored as ferritin or released into the fetal circulation via ferroportin on the basolateral side of the placental syncytiotrophoblast(Abboud and Haile, 2000; Harris, 1992). Ferrous iron thus released is converted to its ferric form by a ferroxidase(Chen et al., 2010).

Hepcidin in pregnancy

During pregnancy, maternal hepcidin levels are determined by the maternal iron status and fetal demands (Koenig et al., 2014). Studies have shown that hepcidin levels are low in the third trimester, when there is increased transfer of maternal iron to the fetus(Gyarmati et al., 2011; Dao et al., 2013). Some studies have shown correlation between maternal hepcidin levels and markers of iron status (Schulze et al., 2008) (Rehu et al., 2010;van Santen et al., 2013).

A meta-analysis on hepcidin and its role in pregnancy included 10 human and 6 animal studies (Koenig et al., 2014). The human studies comprised both cross-sectional and longitudinal studies, mostly involving healthy pregnancies with few complications. Hepcidin levels have been shown to be lower in pregnant women compared to non-pregnant healthywomen(Rehu et al., 2010b). Lowest levels of hepcidin have been reported during the third trimester. This correlates with the high uptake of iron from the maternal to the fetal side (Gyarmati et al., 2011b; Finkenstedt et al., 2012; van Santen et al., 2013b; Dao et al., 2013). Maternal hepcidin levels have been shown to correlate with markers of iron status (Schulze et al., 2008; Rehu et al., 2010c; van Santen et al., 2013b).In uncomplicated pregnancies, it was shown that maternal hepcidin levels during gestation did not correlate with inflammatory markers (Schulze et al., 2008; van Santen et al., 2013b). However, in the setting of obesity(Dao et al., 2013) or preeclampsia (Toldi et al., 2010), serum hepcidin levels were found to correlate with levels of C-reactive protein (CRP), an inflammatory marker.

Gestational diabetes mellitus (GDM)

Defects in insulin production or insulin action or both lead to diabetes mellitus (Alberti and Zimmet, 1998). Globally, about 422 million people are estimated to have diabetes mellitus, with the number predicted to increase to 592 million people by 2030. India has the world's second largest diabetic population with about 62 million diabetics (Anjana et al., 2011).

Gestational diabetes mellitus (GDM) is defined as diabetes diagnosed in the second or third trimester of pregnancy that is not clearly either type 1 or type 2 diabetes (ADA, 2016). The prevalence of GDM in India has been estimated to be between 3%-21% (Kaveeshwar and Cornwall, 2014; Zhu and Zhang, 2016). The large variation in these prevalence figures has been suggested to be due to the use of different diagnostic criteria, differences in lifestyles and food habits of the subjects, etc(Rajput et al., 2013). In Tamil Nadu, the prevalence has been estimated to about 13.8% [urban-17.8%; semiurban-13.8%; rural-9.9%] (Anjana et al., 2011).

The screening of GDM is usually done between 24 to 28 weeks of gestation (Sacks et al., 2012). One of the most commonly used criteria to diagnose GDM is InternationalAssociation of the Diabetes and Pregnancy Study Groups (IADPSG)criteria (ADA, 2016); these comprise a fasting plasma glucose levels equal to or greater than 92 mg/dL or a 1-hour (post oral glucose load of 75 g) plasma glucose value equal to or greater than 180 mg/dL or a 2-hour (post oral glucose load) plasma glucose value equal or greater than 153 mg/dL(ADA, 2016).

GDM is mainly due to insulin resistance that starts during the second trimester and is present through the rest of pregnancy (Zhu and Zhang, 2016). Insulin resistance has been attributed mainly to placental hormones and maternal adiposity (Kaaja and Rönnemaa, 2008). Human placental lactogen [HPL], human chorionic somatomammotropin [HCS], estrogen, progesterone, prolactin and cortisol are placental hormones that do not cross the placenta. Higher concentrations of these hormones due to increases in placental size in the later weeks of gestation can cause increases in insulin resistance (Kaaja and Rönnemaa, 2008; Kühl, 1998).

Gestational diabetes mellitus is the most common metabolic disorder during pregnancy (Buchanan et al., 2012). It is associated with maternal and fetal complications. Though the exact mechanism is not known, iron overload has been reported to induce β-cell toxicity and to impair glucose metabolism (Lao et al.,2001;Ferrannini, 2000). High iron and low insulin levels have been reported in patients with GDM (Kaygusuz et al., 2013). Iron has been reported to be a strong pro-oxidant that can affect insulin synthesis in the pancreas (Rajpathak et al., 2009).

The link between iron homeostasis and diabetes mellitus was first noted in patients with hemochromatosis, who were found to have a high incidence of diabetes mellitus (Fernández-Real et al., 2005; Jiang et al., 2004). Frequent blood donations have been shown to decreased risk of developing diabetes mellitus (Fernández-Real et al., 2005; Jiang et al., 2004; Swaminathan et al., 2007). Treatment of patients with thalassemia, who have iron overload, with iron chelators has decreased the incidence of diabetes mellitus in this group (Gamberini et al., 2008) and also decreased diabetes-related vascular complications (Nitenberg et al., 2004).

Serum ferritin is a glycated L-subunit of ferritin that is poor in iron (Wang et al., 2010). It is elevated in conditions of iron overload. Serum ferritin levels have been reported to be elevated in subjects with GDM in studies done in Turkey, Iran, Denmark and USA (Derbent et al., 2013; Amiri et al., 2013; Bowers et al., 2016; Chen et al., 2006).There are no such published studies from India.

THE STUDY

HYPOTHESIS

Serum ferritin levels may be increased in Indian women with gestational diabetes mellitus (GDM).

OBJECTIVES OF THE STUDY

a. To estimate serum ferritin in subjects with and without GDM
b. To estimate various iron-related parameters in subjects with and without GDM
c. To compare the above parameters in those with and without GDM
d. To determine the outcome of pregnancy in subjects with and without GDM and correlate these with estimated parameters

MATERIALS

EQUIPMENT USED

1. Elix and Milli-Q ultrapure water system (Millipore, USA)
2. -70^{O}C freezer (Thermo Scientific, Massachusetts, USA)
3. Table-top refrigerated centrifuge (MPW R 350, MPW Poland)
4. Micro-plate reader (Model 680, Bio-Rad laboratories, Inc., UK)

CHEMICALS AND REAGENTS USED FOR HEPCIDIN ESTIMATION

These were obtained from Peninsula Laboratories (San Carlos, USA) and consisted of the following:

1. Lyophilized standard, anti-serum against hepcidin, biotinylated peptide
2. Enzyme immune-assay buffer, streptavidin-horseradish peroxidase (HRP), substrate solution (TMB – 3, 3', 5, 5'–tetramethylbenzidine solution) and stop solution (2N HCl)

OTHER MATERIALS USED

1. Plain red vacutainer tubes for blood collection (BD Biosciences, Plymouth, UK)
2. Micro-tubes (Tarson Products Private Limited, Kolkata, India)
3. Micro-tips (Tarson Products Private Limited, Kolkata, India)

METHODS

The Institutional Review Board (IRB) at Christian Medical College (CMC), Vellore, India, approved this study (IRB Min No 10423 dated 05.12.2016) (included as Appendix 1).

SUBJECTS

Primigravidae, who attended the antenatal clinic of the Community Health and Development (CHAD) Hospital in CMC, Vellore, and who were referred for an oral glucose tolerance test (OGTT) (75 gm glucose) as part of their routine antenatal check-ups, were recruited for this study. The period of recruitment was from January 2017 to July 2018.The inclusion criteria were as follows:

Inclusion criteria

Primigravidae

a. who gave consent to participate in the study
b. who had a packed cell volume (PCV) equal to or greater than 33.0% or haemoglobin levels equal to or more than 11 g/dL in the first trimester
c. with serum C-reactive protein (CRP) level less than or equal to 12 mg/L (Abbassi-Ghanavati et al., 2009; Nakishbandy and Barawi, 2014)

Exclusion criteria

Primigravidae who

a. declined to give consent to participate in the study

b. had PCV less than 33.0% or haemoglobin levels less than 11 g/dL in the first trimester
c. had serum CRP levels greater than or equal to 12 mg/L
d. were known to have diabetes mellitus or hypertension
e. had any pregnancy-related complication, such as chronic infective or inflammatory states or pregnancy-induced hypertension or preeclampsia or multiple gestation
f.

INFORMED CONSENT

When potential participants were identified, based on the inclusion and exclusion criteria listed above, the study was explained to them. They were also provided with an information sheet printed in English or Tamil, depending on their preference. Written informed consent was obtained from each participant. The format for the informed consent form and the information sheet used is included as Appendix 2.

CLINICAL AND DEMOGRAPHIC DATA

Relevant clinical and demographic data were collected, using a proforma (included as Appendix 3). Each participant's date of last menstrual period was used to calculate gestational age. The haematocrit (Hct) values were obtained from their hospital records.

Blood pressure was measured in each subject, using an automatic blood pressure apparatus. Each woman's mid-arm circumference was measured, by using a non-

stretchable measuring tape, at a point midway between the acromion process of the left scapula and the olecranon process of the ulna on the same side.

All the primigravidae had been prescribed ferrous sulphate (150 mg per day) and folic acid tablets (2.5 mg per day) from the time of their first antenatal visit, as per National Nutritional Anemia Control Programme (NNACP) guidelines (Kumar, 1999). The date when iron and folic acid supplements were prescribed was noted from hospital records. At the time of recruitment into the present study, they were asked whether they had been taking the tablets regularly. Information on outcomes of pregnancy was obtained from hospital records and by contacting the subjects by phone.

CALCULATION OF SAMPLE SIZE

Information from a study by Derbent et al, (2013) was used to calculate sample size. To show a difference of 11.45 units of serum ferritin between women with and without GDM, with 90% power and 5% level of significance, 22 samples were required in each group (standard deviation of 13.37 in women with GDM and 9.66 in women without GDM). The details of this calculation are shown below.

	Derbent et al 2013
Standard deviation in women with GDM	13.37
Standard deviation in women without GDM	9.66
Mean difference	11.45

Effect size	0.9943
Alpha error (%)	5
Power (1- beta) %	90
1 or 2 sided	2
Required sample size per group	**22**

Formula:

$$\frac{(Z_{\alpha/2} + Z_{1-\beta})^2 * 2 * sd^2}{d^2}$$

Where,

$Z_{\alpha/2}$ is 5% level of significance

$Z_{1-\beta}$ is the 80% power

sd = standard deviation

d = 21.7 (mean change)

SAMPLE COLLECTION

A blood sample (fasting) (8 ml) was collected from each subject at the time of her OGTT. These were collected in plain red BD vacutainer tube, by venepuncture of the cubital vein.

PROCESSING AND STORAGE OF SAMPLES

The blood samples obtained were subjected to centrifugation at 2500*g* for 10 minutes, within 2 hours of sample collection. The serum obtained was divided into multiple aliquots and stored at -70^{O}C till used for analyses. They were thawed on ice, when required for estimations of serum ferritin, iron, CRP and hepcidin.

ESTIMATION OF SERUM FERRITIN

Serum ferritin was measured in Department of Clinical Biochemistry, CMC, Vellore. This is a routine test offered by this laboratory.

Analyzer used

Siemens, ADVIA, Centaur System, XPi, UK

Principle of the method (two-site sandwich immunoassay using direct chemiluminescence technology)

In this method, two anti-ferritin antibodies were used. The first antibody, which is a monoclonal mouse anti-ferritin antibody in the solid phase, was covalently attached to paramagnetic particles. The second antibody was a polyclonal goat anti-ferritin antibody linked with acridinium ester. These antibodies were sequentially added to the reaction container. They bound to the ferritin present in the sample. On adding a substrate (0.1N nitric acid, 0.5% hydrogen peroxide in alkaline medium) that excites acridinium, photons were released, which were measured as relative light units (RLU). The amount of RLU detected was directly proportional to the amount of ferritin present in the sample.

ESTIMATION OF SERUM IRON

Estimation of serum iron was done in the Department of Clinical Biochemistry, CMC, Vellore. This is a routine test offered by this laboratory.

Analyzer used

Roche Cobas C720 modular analyzer from Roche Diagnostics, Gmbh, Mannheim

Principle (colorimetric method)

In the acidic medium provided, ferric iron was released from transferrin. Ascorbate was added to reduce ferric iron into ferrous form. Ferrous iron bound with a dye, ferrozine, to form a purple coloured complex, which was measured at 560 nm. The intensity of the purple colour that developed was directly proportional to the amount of iron in the sample.

ESTIMATION OF TOTAL IRON-BINDING CAPACITY (TIBC)

Estimation of TIBC was done in the Department of Clinical Biochemistry, CMC, Vellore. This is a routine test offered by this laboratory.

Analyzer used

Roche Cobas C720 modular analyser from Roche Diagnostics, Gmbh, Mannheim

Principle (colorimetric method)

To a known amount of reagent solution (consisting of an alkaline buffer, containing known concentrations of iron and ferrozine), the serum sample was added. At alkaline pH, iron in the reagent binds with unoccupied sites on transferrin, leaving unbound iron free. The free iron complexes with ferrozine to give a purple complex, the intensity of colour of which was measured at 560 nm. The intensity of the purple

colour that developed was directly proportional to the amount of unbound iron. The difference between the amount of iron added and the unbound iron was taken to be the unbound iron binding capacity (UIBC). The sum of serum iron and UIBC represented total iron binding capacity (TIBC).

CALCULATION OF TRANSFERRIN SATURATION

Transferrin saturation was calculated as the percentage of the ratio of serum iron to TIBC, as shown in the formula below:

$$\text{Transferrin saturation (\%)} = (\text{Serum iron}/\text{TIBC}) \times 100$$

ESTIMATION OF SERUM C-REACTIVE PROTEIN

Estimations of CRP were done in the Department of Clinical Microbiology, CMC, Vellore. This is a routine test offered by this laboratory.

Analyzer used

BN Prospec, Siemens, Gmbh, Mannheim, Germany

Principle (nephelometry)

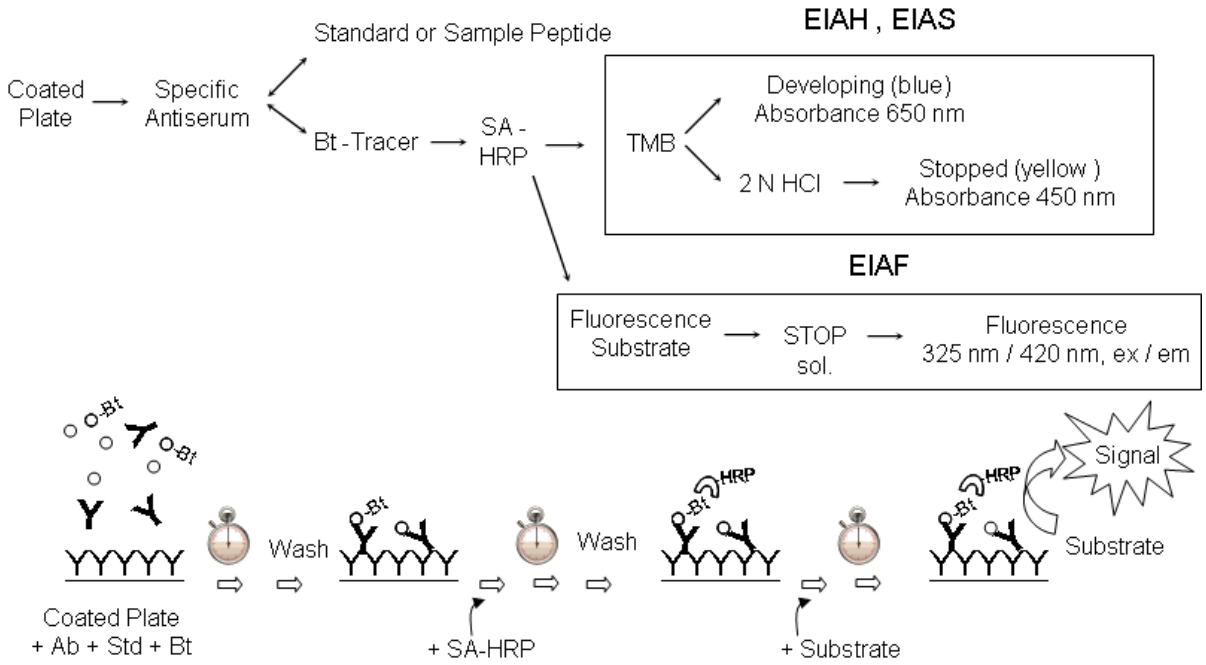
Polysterene particles (coated with antibodies against CRP) aggregated with serum CRP, when a serum sample was added to the reaction chamber. This aggregation led to scattering of light, when light was passed through the reaction chamber. The amount of light scattered was measured by a sensor and was directly proportional to the amount of CRP present in the sample.

ESTIMATION OF SERUM HEPCIDIN

ELISA kit and reagents for hepcidin estimation were purchased from Peninsula Laboratories, (San Carlos, USA).

Principle of the method

The estimation of hepcidin was based on a competitive immunoassay. Hepcidin in the sample competed with a biotinylated tracer to bind to the antibody against hepcidin. The amount of biotinylated tracer that bound to the antibody was inversely proportional to the amount of hepcidin in the sample.



Source: Product information sheet of the hepcidin kit (Hepcidin-25 [for serum and plasma samples]) Peninsula Laboratories (Cat No S-1337)

Components of the kit

1. 96-well plate
2. Biotinylated peptide, in the form of a lyophilised powder
3. Hepcidin peptide (lyophilised powder) (1µg)

4. Rabbit antiserum against hepcidin 25(lyophilised powder)
5. Enzyme immunoassay buffer (50mL)
6. Standard diluents (peptide-free human serum) (8mL)
7. Streptavidin horseradish peroxidase (100µL)
8. Substrate solution (TMB 3,3',5,5'-tetramethyl benzidine)(11mL of TMB and hydrogen peroxide)
9. 2N HCl (hydrochloric acid) (15mL) (stop solution)

As per manufacturer's instructions, enzyme immunoassay buffer, streptavidin-HRP substrate and stop solution were stored in a refrigerator (2 to 8^{o}C). The diluents for the standard, lyophilised standard, anti-serum and biotinylated peptide were stored at -20^{o}C. The stability of these reagents was 1 year, under conditions listed above.

Preparation of reagents

The unopened kit and reagents were brought to room temperature, before preparation of working reagents and samples.

1. Stock standard: To 1µg of lyophilised standard, 1mL of standard diluent was added and mixed thoroughly, using a vortex mixer.

Standard	ng/mL	Range : 0.02 to 25ng/mL
Stock	1000	
S_1	25	Added 5µL stock + 195µL diluents
S_2	6.25	Added 40µL (S_1)+ 40µL diluents
S_3	1.56	Added 40µL (S_2)+ 40µL diluents
S_4	0.39	Added 40µL (S_3)+ 40µL diluents

S_5	0.10	Added 40µL (S_4)+ 40µL diluents
S_6	0.02	Added 40µL (S_5)+ 40µLdiluents
S_0	0.00	120µL diluents

2. Samples: The samples in the study were diluted 1 in 10 (12µL of sample + 108 µL of standard diluent). Dilutions were carried out to bring the concentration of the analyte within the analytical measurement range.

3. Enzyme immunoassay buffer (EIA buffer): EIA buffer (50mL) was diluted to 1000mL, using sterile deionised water (18Mohm).

4. Anti-serum: To the lyophilised anti-serum powder, 5mL of EIA buffer was added and mixed well.

5. Biotinylated tracer (bt tracer): To the lyophilisedBt-tracer powder, 5mL of EIA buffer was added and mixed well.

6. Streptavidin- HRP: This was diluted 1in 200 with EIA buffer (60 µL of streptavidin-HRP made up to 12 mL with EIA buffer), and mixed well.

Layout of a 96-well coated micro-well plate for the hepcidin assay

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	T_1	T_1	T_9	T_9	T_{17}	T_{17}	T_{25}	T_{25}	T_{33}	T_{33}
B	S_1	S_1	T_2	T_2	T_{10}	T_{10}	T_{18}	T_{18}	T_{26}	T_{26}	T_{34}	T_{34}
C	S_2	S_2	T_3	T_3	T_{11}	T_{11}	T_{19}	T_{19}	T_{27}	T_{27}	T_{35}	T_{35}
D	S_3	S_3	T_4	T_4	T_{12}	T_{12}	T_{20}	T_{20}	T_{28}	T_{28}	T_{36}	T_{36}
E	S_4	S_4	T_5	T_5	T_{13}	T_{13}	T_{21}	T_{21}	T_{29}	T_{29}	T_{37}	T_{37}
F	S_5	S_5	T_6	T_6	T_{14}	T_{14}	T_{22}	T_{22}	T_{30}	T_{30}	T_{38}	T_{38}
G	S_6	S_6	T_7	T_7	T_{15}	T_{15}	T_{23}	T_{23}	T_{31}	T_{31}	T_{39}	T_{39}
H	S_0	S_0	T_8	T_8	T_{16}	T_{16}	T_{24}	T_{24}	T_{32}	T_{32}	T_{40}	T_{40}

B- blank, S- standard, T- test samples

Assay procedure

Step 1: 25µL of antiserum was added to each well; 25µL of EIA buffer was added to the blank well. These were incubated for 1hour at room temperature.

Step 2: 50µL of diluted standard/ sample was added to respective wells. To the blank well, 50µL of diluent was added. The plate was incubated for 2hours at room temperature.

Step 3: 25µL of reconstituted Bt-tracer was added to each well.

Step 4: Using its acetate plate cover (provided with the kit), the microplate was sealed and incubated overnight, at 4^oC in a refrigerator.

Step 5: The microplate was taken out of the refrigerator the next day and allowed to come to room temperature.

Step 6: Each well was carefully washed, with 300µL of EIA buffer. This was done 5 times.

Step7: 100µL of streptavidin-HRP solution was added to each well. The plate was incubated at room temperature for 1hr.

Step 8: At the end of the incubation, each well was washed with 300µL of EIA buffer. This was done 5 times.

Step 9: 100µL of TMB (substrate solution) was added to each well. The reaction mixture was incubated at room temperature for 60mins.

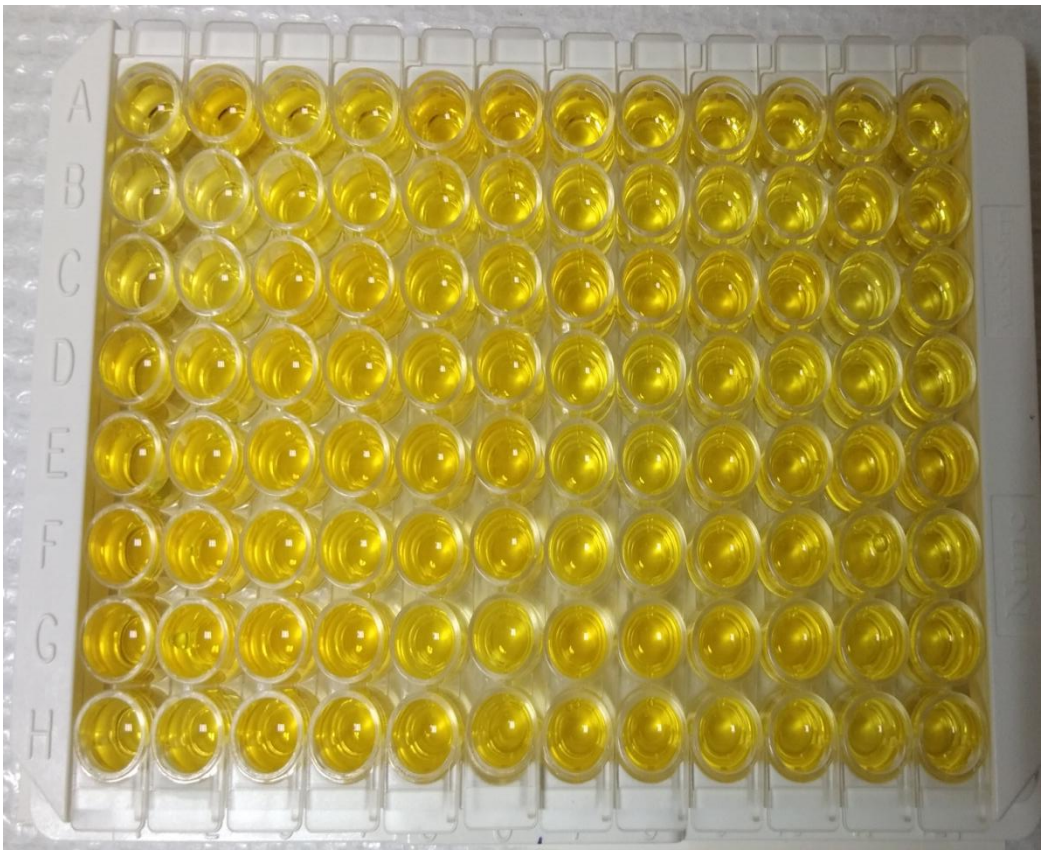
Step 10: During development of a blue color that ensued, readings of the wells in the plate were taken at 650 nm,at 15, 30, 45, and 60 minutes after addition of TMB.

Step 11: At the end of 60 minutes, 2N HCl (100µL per well) was added to terminate the reaction.

Step 12: Readings were then taken at 450 nm, at 3, 6, and 10 mins after terminating the reaction.

The intensity of colour in each plate was measured as its optical density (OD),using the software for the microplate manager of the ELISA plate reader.

Image of the microtitre plate after termination of the reaction

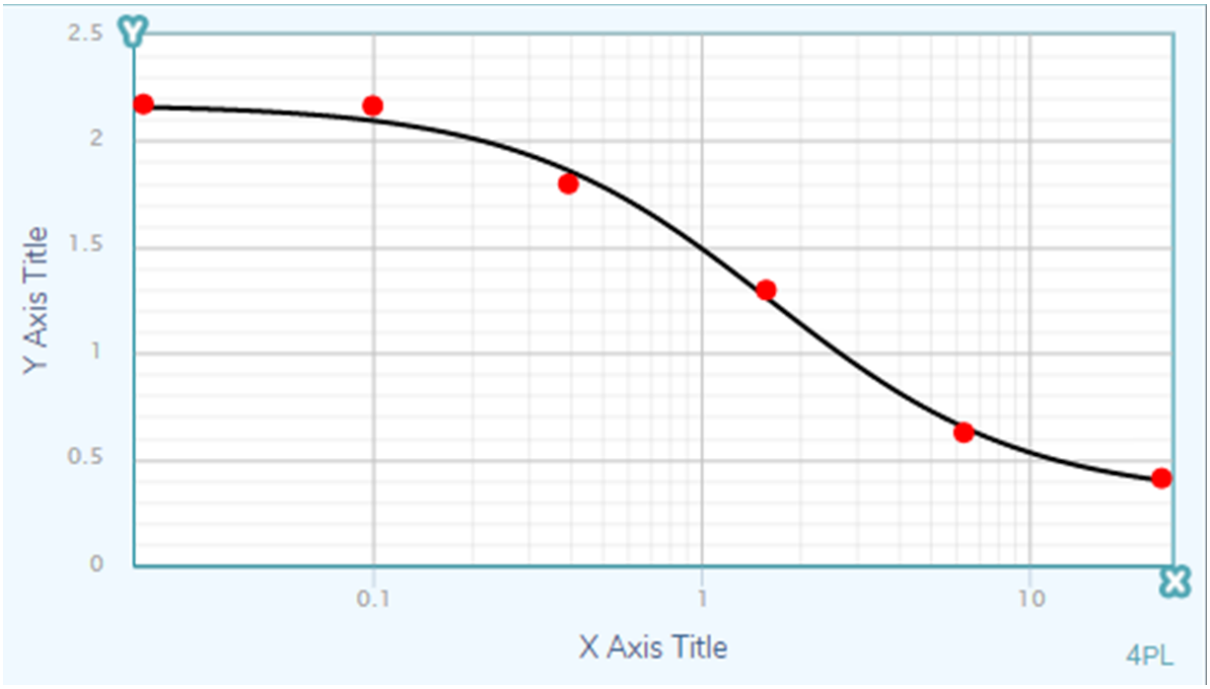


All standards and samples were estimated in duplicate; the average of the OD readings was used for purposes of calculations. The OD values obtained for the standards were used to plot a standard curve, by 4-parameter logistic regression. A semi-log scale was used (on Microsoft Office Excel 2010). The concentrations of standards (ng/mL) were plotted on the X-axis and the OD readings on Y-axis. Values for serum hepcidin levels were calculated after adjusting for sample dilutions (dilution factor of 10).This was done, using four parameter logistic regression analysis, by the following equation:

$$Y= [(a-d) + \{1+ (x/c)\ b\}] + d$$

The values **a** (maximum value of OD in the standard curve), **b** (slope), **c** (point of inflection (IC50)), **d** (minimum value of OD in the standard curve) in the equation were used to get the standard curve to fit the data, as closely as possible.

Standard curve of hepcidin



The concentration of hepcidin (ng/mL) in each sample was calculated, using the following equation:

$$X=[c*\{(x-a)/(d-y)\}^{(1/b)}]$$

DATA ANALYSIS

Data were analyzed using the Statistical Package for Social Sciences (SPSS), version 17. The Kolmogorov-Smirnov test was used to check normality of distribution of data. The independent student's t-test was used to analyze data that was normally distributed and the Mann-Whitney U test for data that had a skewed distribution. For categorical variables, the difference between two groups was analyzed by chi-square test. Pearson's and Spearman's correlation coefficients were used for correlation analysis of normally distributed and skewed data, respectively. In all cases, a p value of less than 0.05 was taken to indicate statistical significance.

RESULTS

Eighty two primigravidae were recruited during the period of the study. Of these, 23 were diagnosed to have GDM (28.04%), based on the results of their OGTT. The remaining 59 had normal results for their OGTT (71.95%). Of the 82 recruited, 49 women (59.75%) delivered their babies in CHAD hospital, 26 (31.70%) delivered in other hospitals and 5 (6.07%) delivered in their homes. At the time of analysis, 2 women (2.43%) were yet to deliver. Two of the women had delivered low-birth weight babies (less than 2500 gm); another baby had respiratory distress initially, but recovered soon.

DISTRIBUTION OF DATA

Data on gestational age at the time of recruitment, values for haematocrit, height, duration for which iron and folic acid supplementation were prescribed, serum iron, TIBC, CRP, gestational age at delivery, birth weight of the newborns and length of the newborns were found to be normally distributed. Data on maternal age, mid-arm circumference, weight, body mass index, plasma glucose values, serum ferritin, serum hepcidin, transferrin saturation and the APGAR score were found to have skewed distributions.

Table 1: Clinical characteristics of subjects in the study

	Controls (N = 59) (median [IQR] /mean ± SD)	**GDM (N = 23) (median [IQR] /mean ± SD)**	**P value**
Age (years)	22 [21-24]	23 [20 - 25]	0.763
Gestational age at time of recruitment (days)	178.59 ± 23	176.48 ± 27.9	0.75
Height (cm)	154.44 ± 7	156.91 ± 6.1	0.123
Weight (kg)	56 [49.4 - 63.5]	62.6 [54 - 734]	0.066
Mid arm circumference (cm)	25 [23-27.5]	26 [24 - 28]	0.226
Haematocrit (%)	36 [34.5 - 37]	36 [35.3 - 37.6]	0.377
Duration for which iron supplements had been prescribed (days)	95.74 ± 41	84.22 ± 40.2	0.255
Body mass index (Kg/m^2)	22.7 [21.2-25.3]	25.8 [22.4-28.1]	0.090

Data were analysed by Mann-Whitney U test or Student's t test, as appropriate.

Maternal ages, gestational ages, height, mid-arm circumference, haematocrit, duration for which iron supplements had been prescribed and body mass index were similar in the control and GDM groups. When asked about regularity of taking the iron supplements, all women said they were compliant. They stated that every morning they took the tablet prescribed, on an empty stomach. The weight of women with GDM tended to be higher than those without GDM (p= 0.066).

Table 2: OGTT resultsin control and GDM subjects

	Controls (N=59) (mean ± SD)	GDM (N = 23) (mean ± SD)	P value
Fasting plasma glucose (mg/dL)	81.93 ± 4.9	92.09 ± 8.4	<0.001
Plasma glucose after 1 hr during OGTT (mg/dL)	135.39 ± 25.3	171.96 ± 40.1	<0.001
Plasma glucose after 2 hr during OGTT (mg/dL)	117 ± 18.3	149.22 ± 32.2	<0.001

Data were analysed by Mann-Whitney U test or Student's t test, as appropriate.

Fasting levels of plasma glucose levels and values at 1 and 2 hours after the glucose load were significantly higher in women with GDM than in those without.

Table 3: Family history of diabetes mellitus

3a:

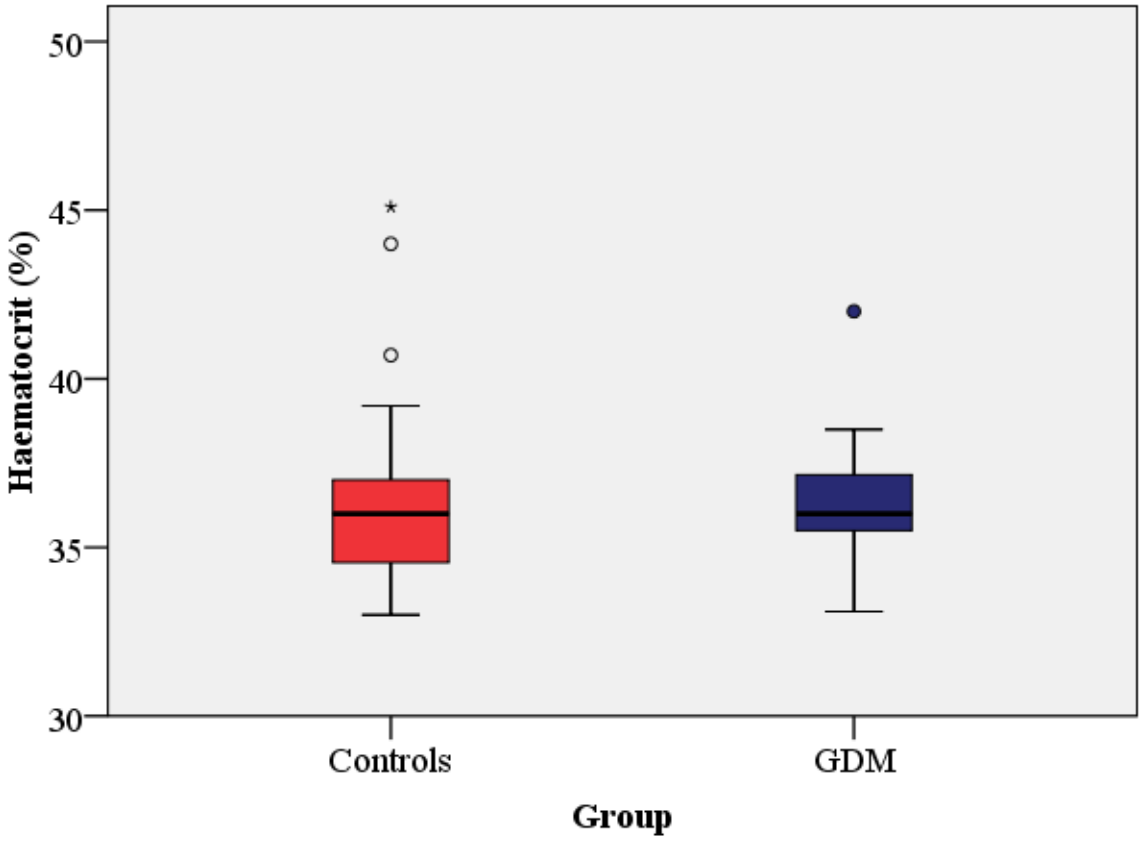
Group	**Family history of diabetes mellitus**	
	Absent	**Present**
Controls (N=59)	27 (45.76%)	32 (54.23%)
GDM (N= 23)	7 (30.43%)	16 (69.56%)

3b:

Group	**Family history of diabetes mellitus**			
	Both parents	**Father**	**Mother**	**No history**
Controls (N=59)	7 (11.86%)	19 (32.20%)	6 (10.16%)	27 (45.76%)
GDM (N=23)	3 (13.04%)	9 (39.13%)	4 (17.39%)	7 (30.43%)

There was no significant difference between the 2 groups, with regard to a family history of diabetes (as assessed by the Chi-square test).

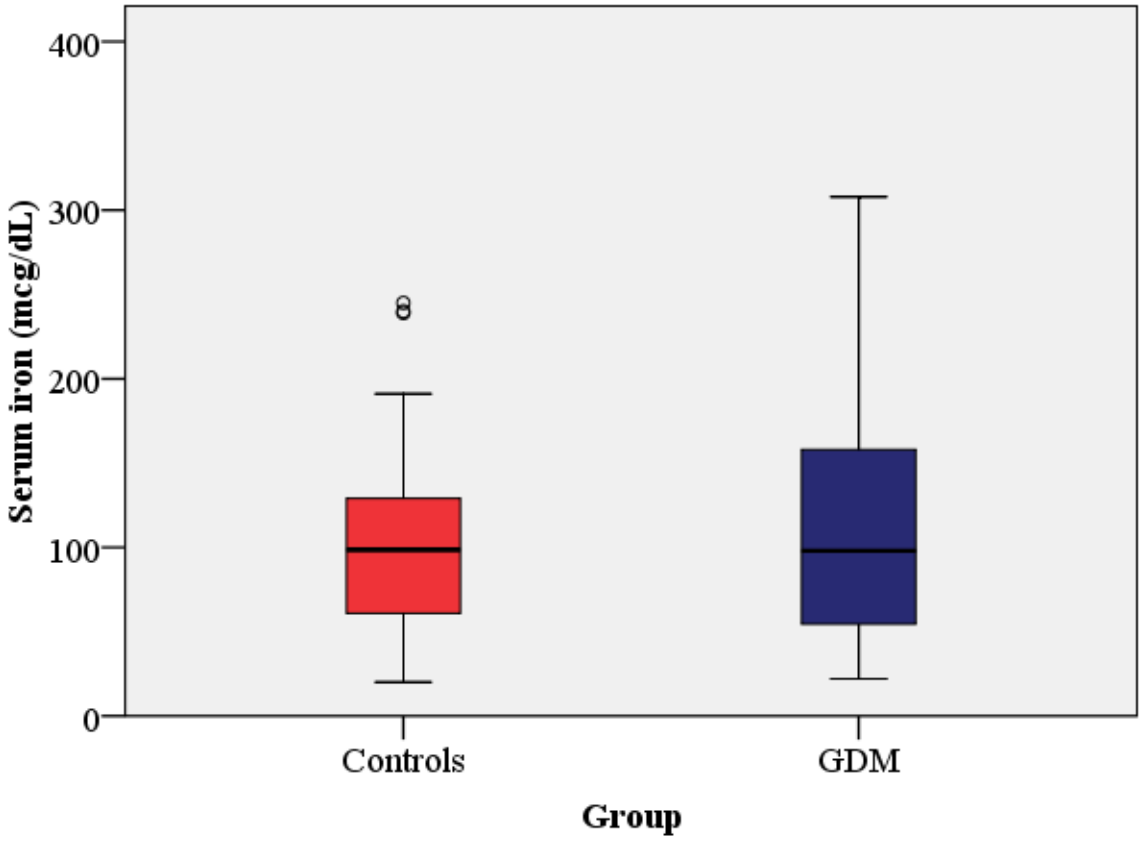
Figure 1: Haematocrit values in control and GDM groups



Data are shown as box and whisker plots, with median and quartiles.

Haematocrit values in the control and GDM groups were similar in both groups (p=0.377).

Figure 2: Serum iron levels in the control and GDM groups

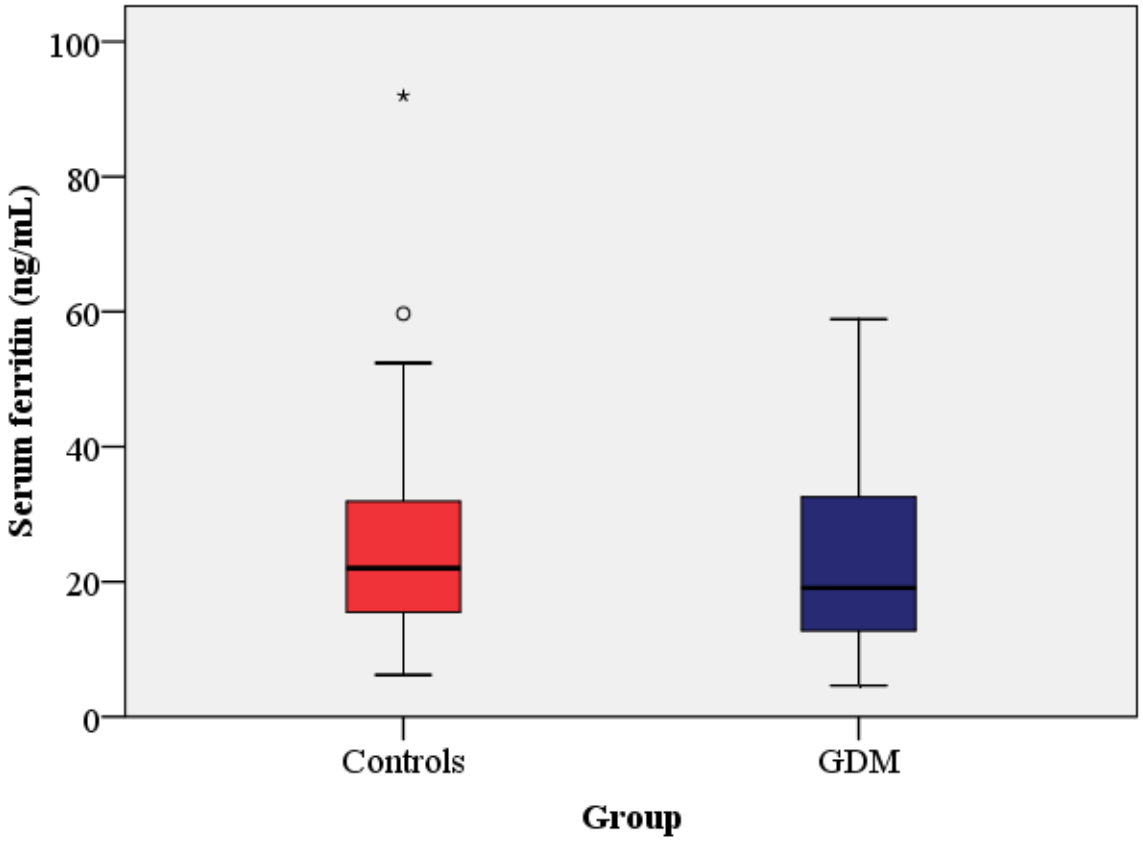


Data are shown as box and whisker plots, with median and quartiles.

Serum iron levels in the control and GDM groups were similar in both groups

($p = 0.987$).

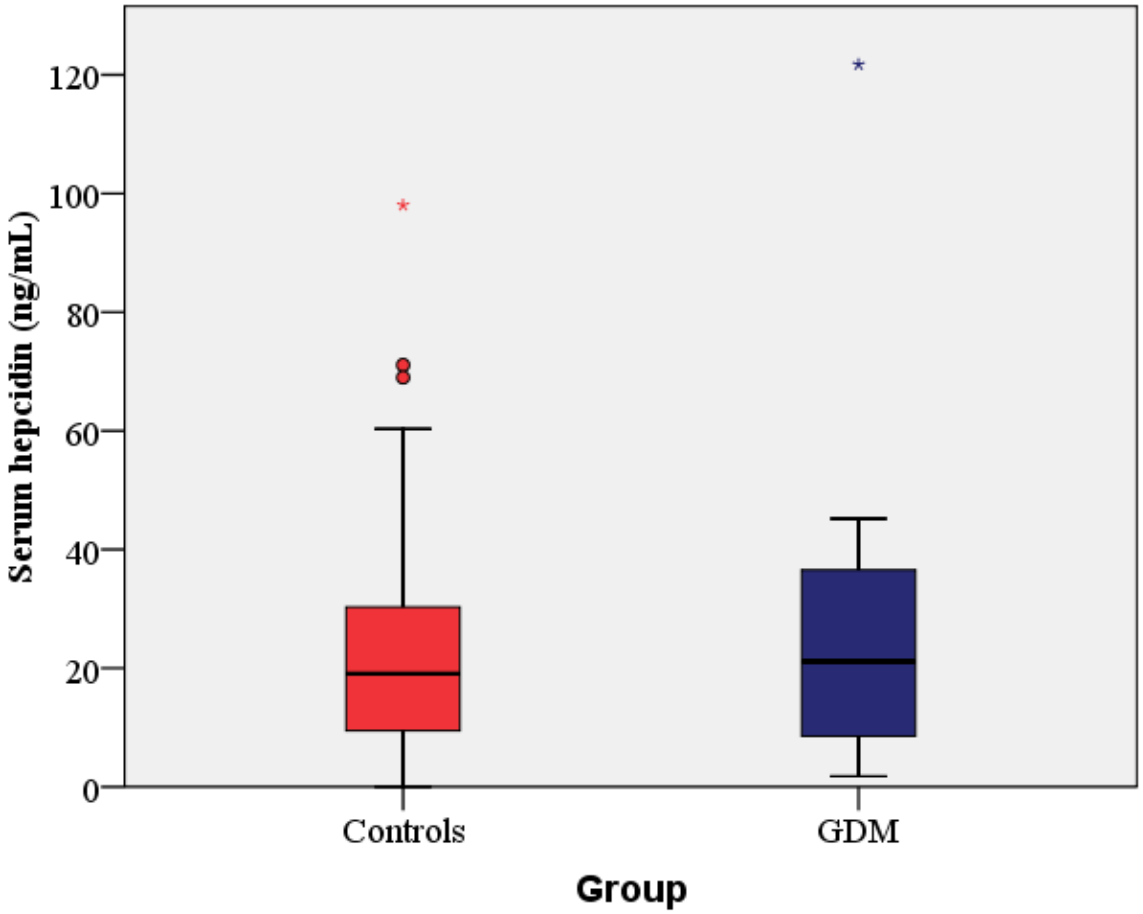
Figure 3: Serum ferritin levels in the control and GDM groups



Data are shown as box and whisker plots, with median and quartiles.

Serum ferritin levels in the control and GDM groups were similar in both groups (p = 0.59).

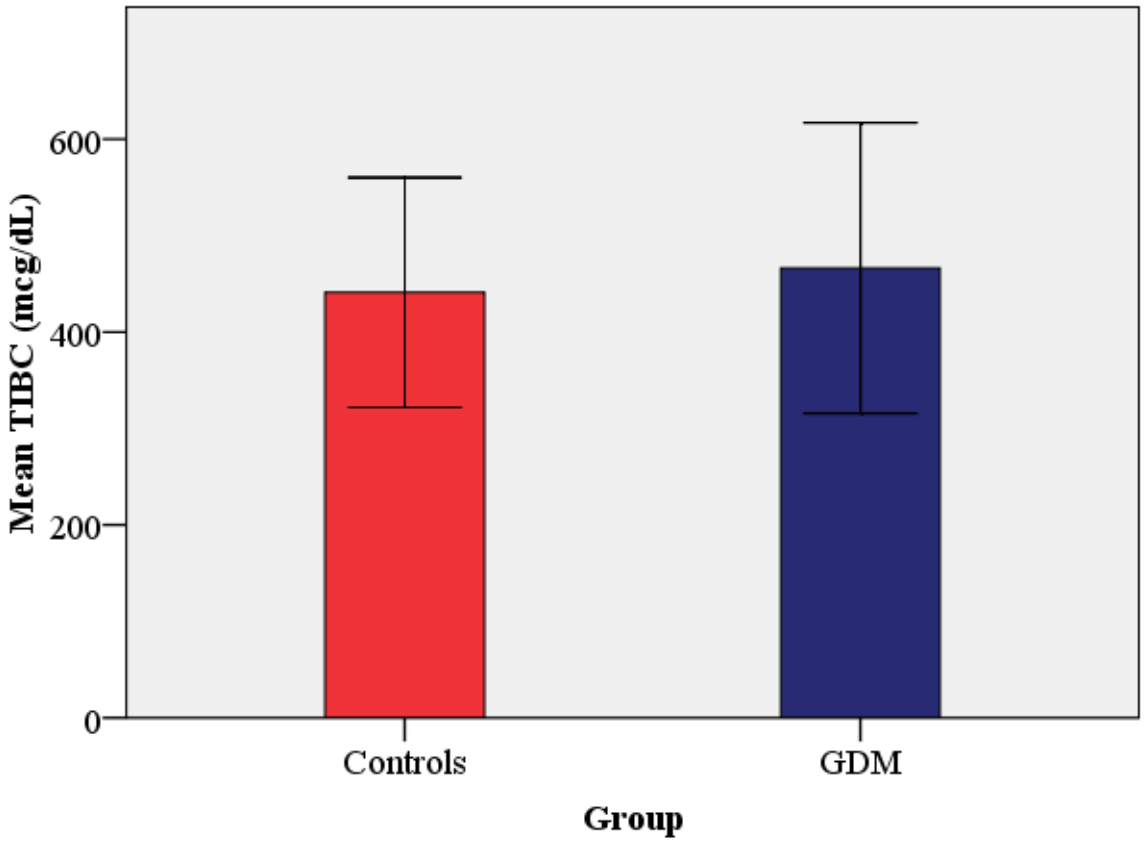
Figure 4: Serum hepcidin levels in the control and GDM groups



Data are shown as box and whisker plots, with median and quartiles.

Serum hepcidin levels in the control and GDM groups were similar in both groups (p = 0.811).

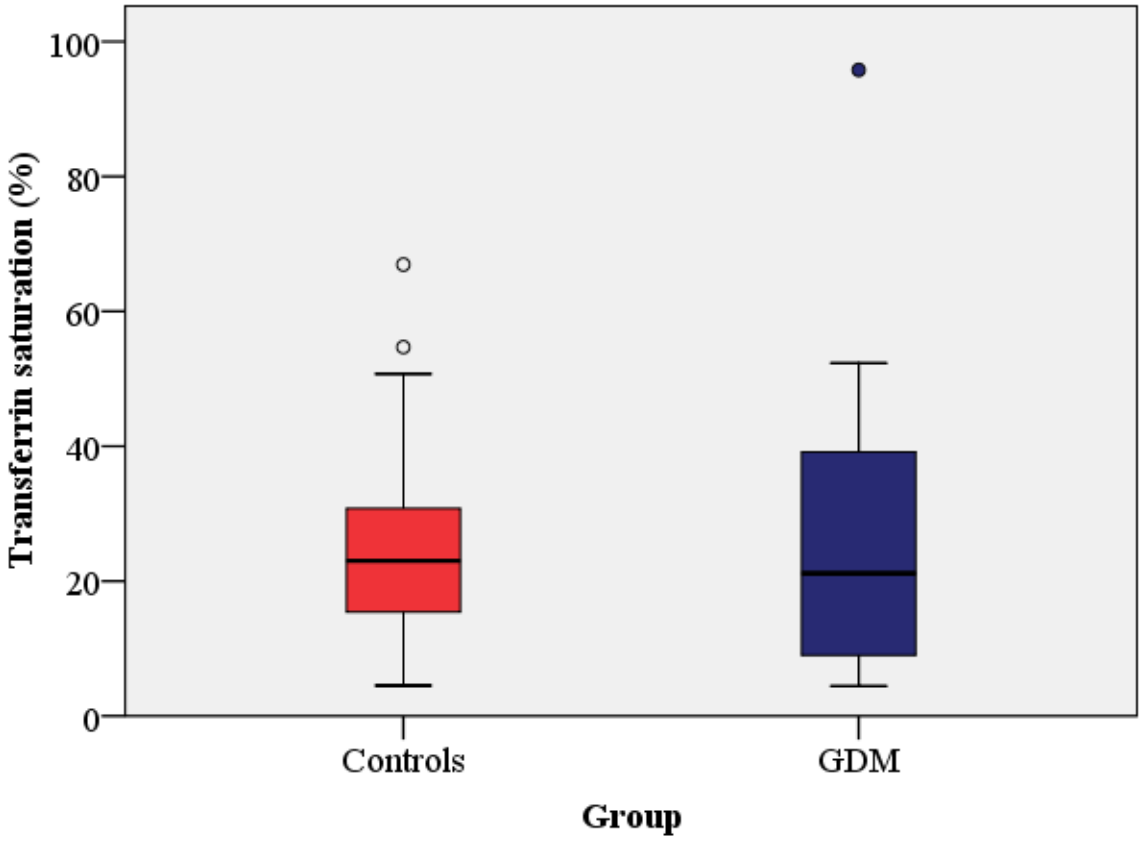
Figure 5: Serum TIBC values in the control and GDM groups



Data are shown as mean ± SD.

Serum TIBC values in the control and GDM groups were similar in both groups (p = 0.167).

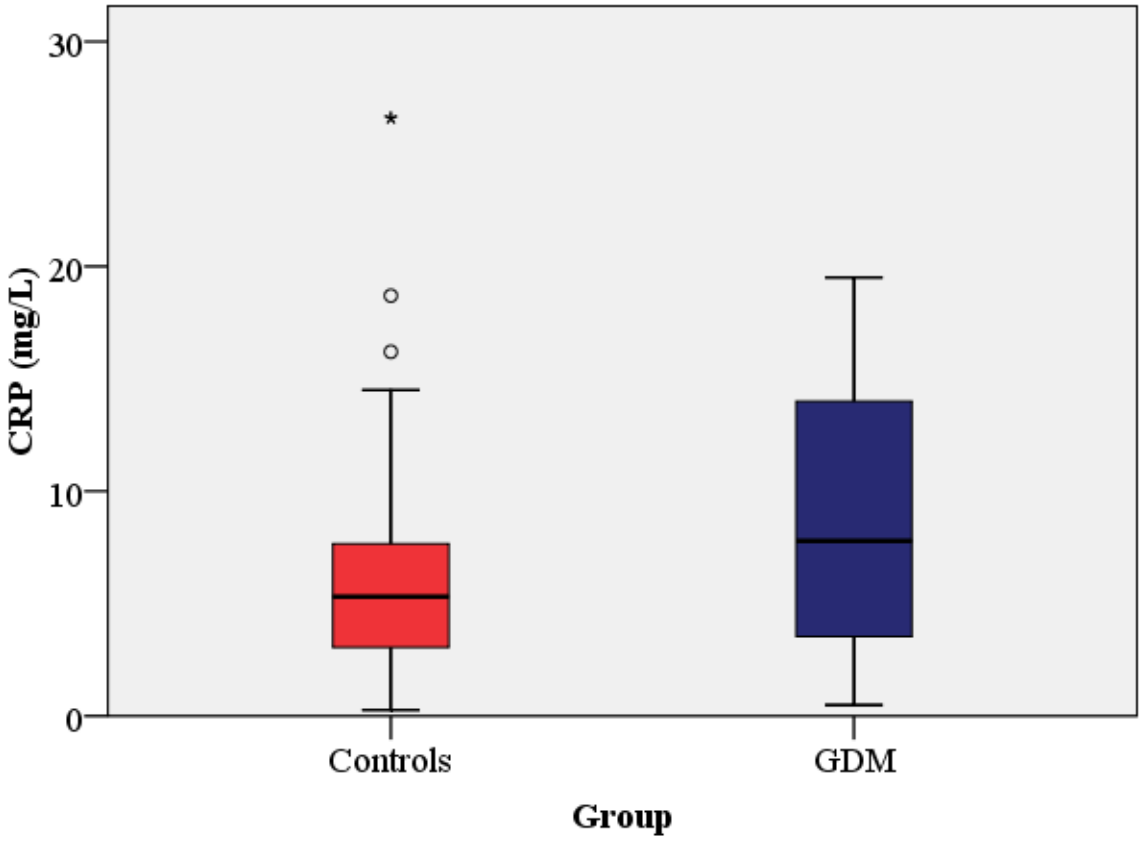
Figure 6: Serum transferrin saturation levels in the control and GDM groups



Data are shown as box and whisker plots, with median and quartiles.

Serum transferrin saturation levels in the control and GDM groups were similar in both groups (p = 1.0).

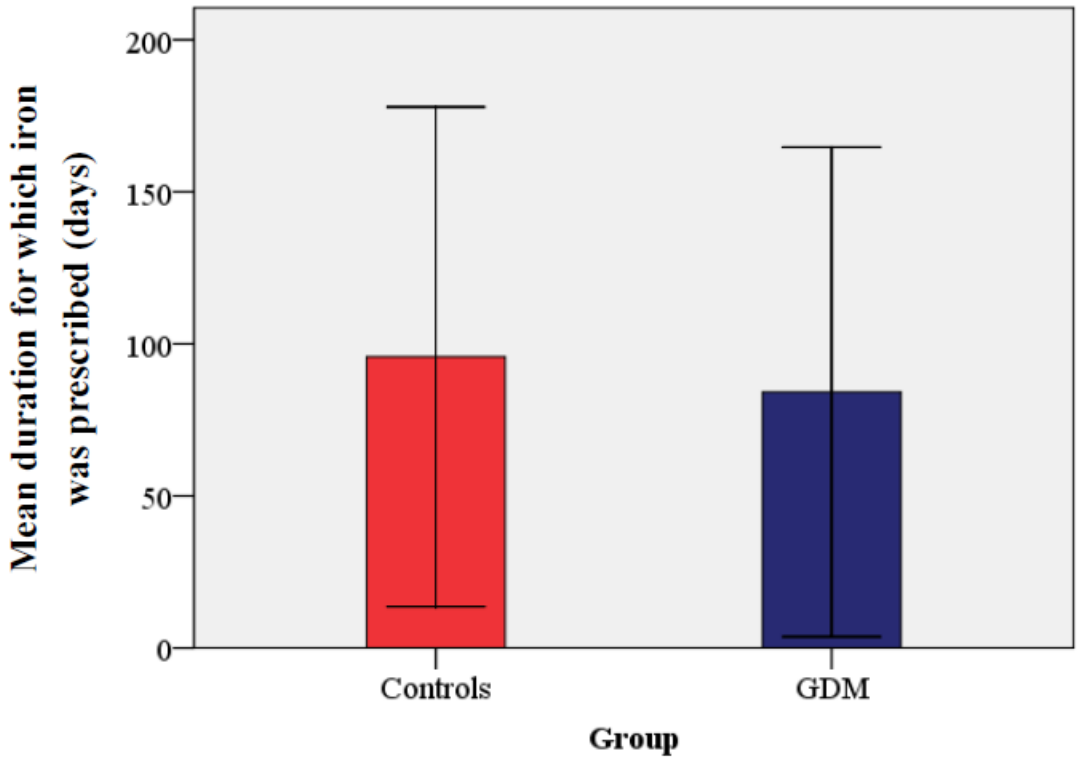
Figure 7: Serum CRP levels in the control and GDM groups



Data are shown as box and whisker plots, with median and quartiles.

Serum CRP levels were not significantly differentin subjects with GDM than those without GDM (p = 0.151).

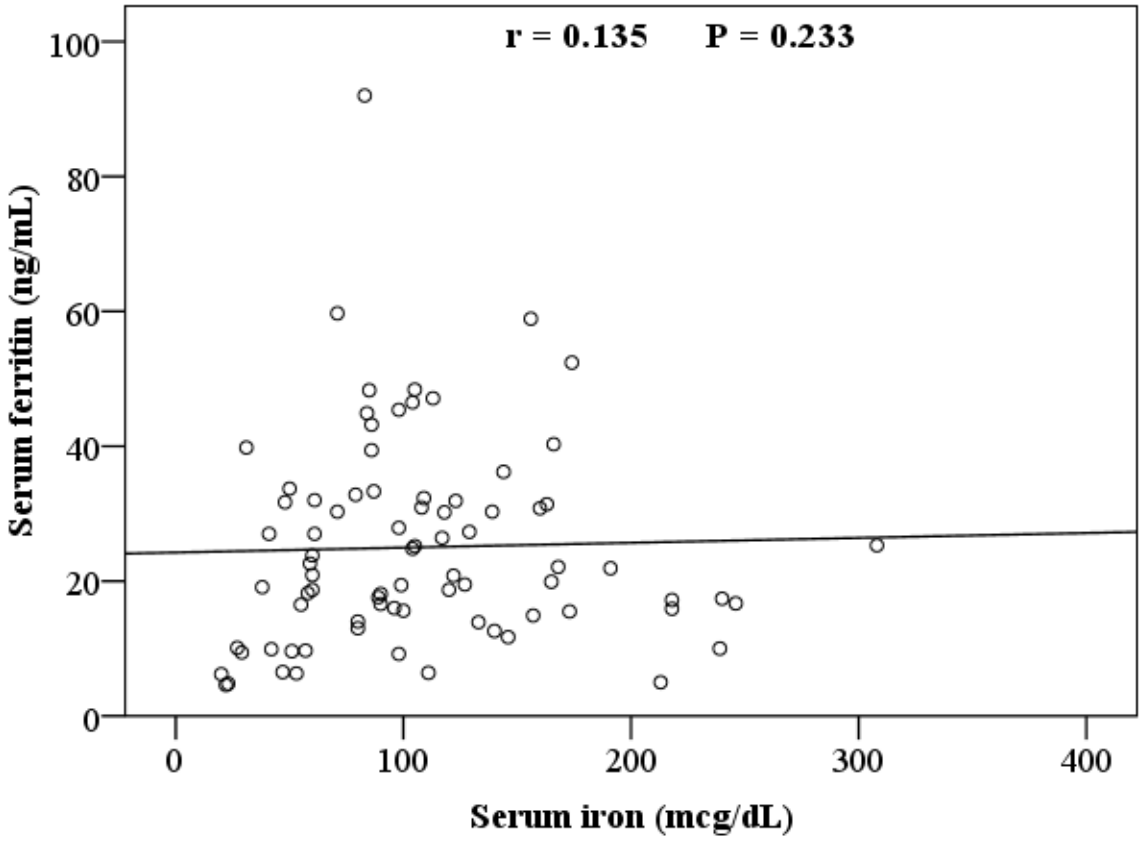
Figure 8: Number of days for which iron was prescribedin the control and GDM groups



Data are shown as mean ± SD.

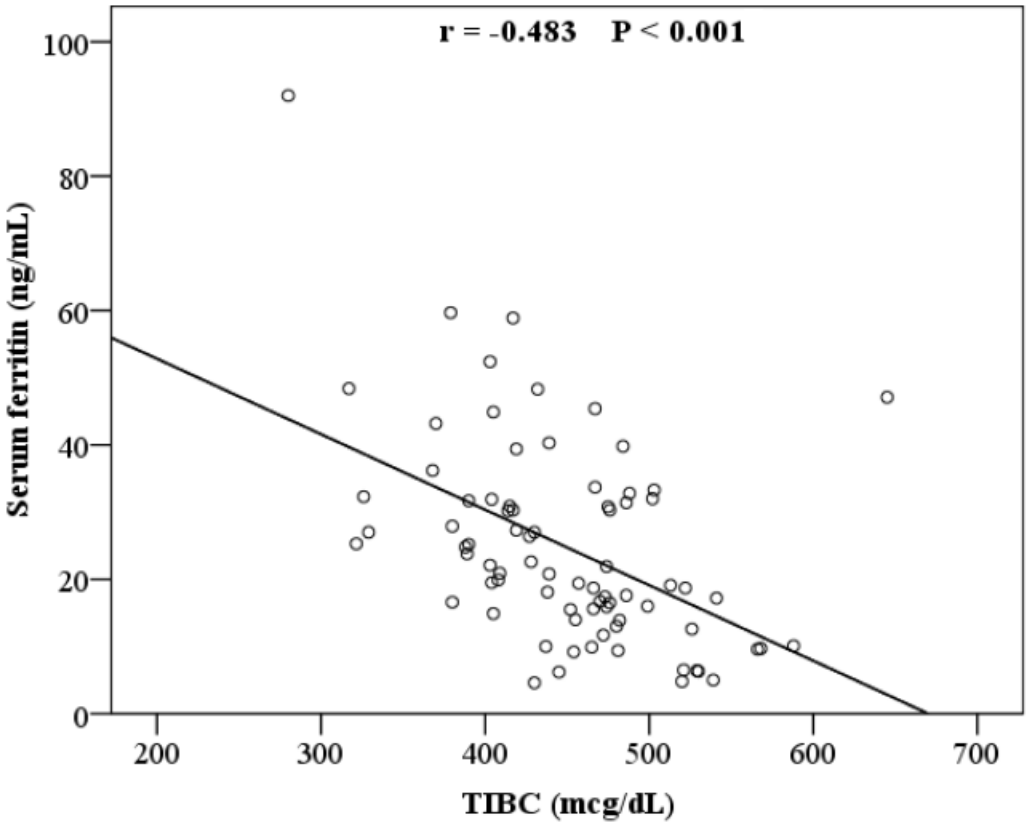
The duration for which iron was prescribedwas similar in both groups (p = 0.255).

Figure 9: Correlation analysis of serum levels of ferritin and iron



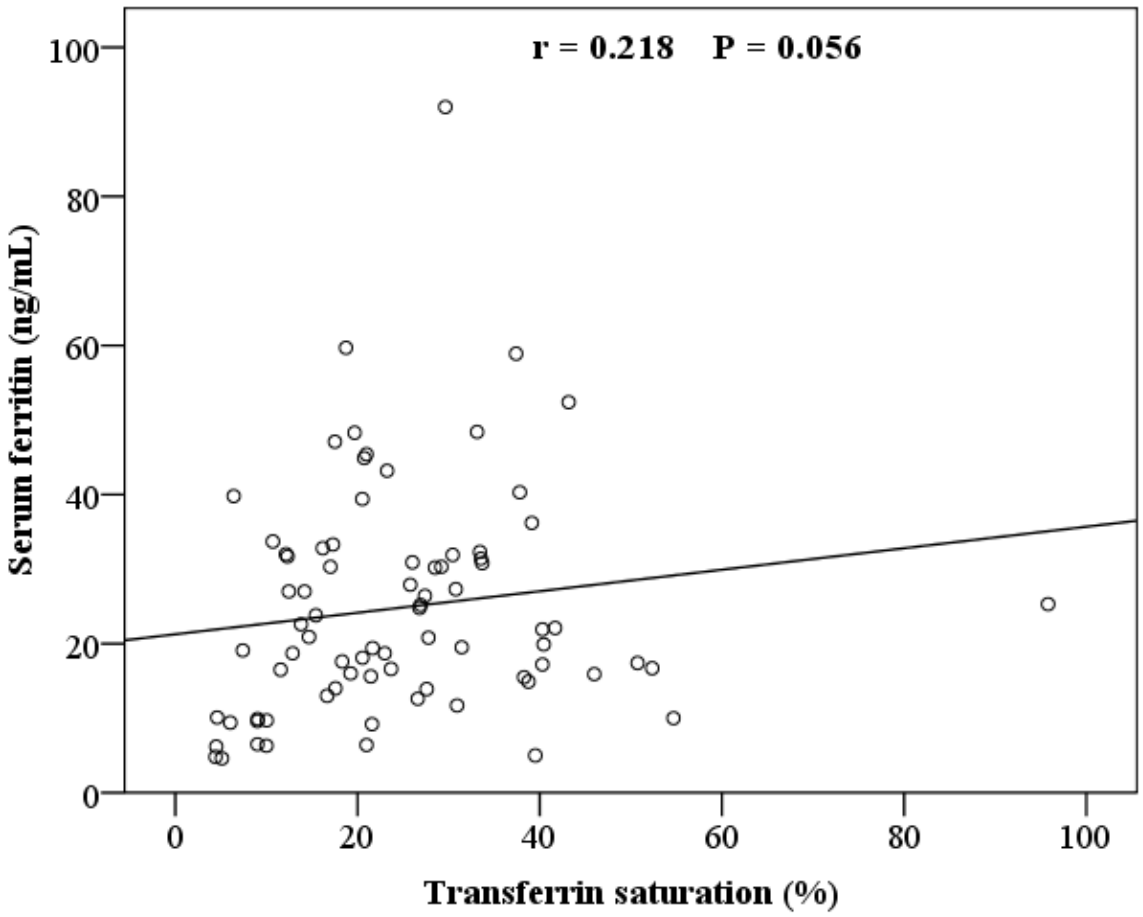
There was no significant correlation between serum levels of ferritin and iron.

Figure 10: Correlation analysis of serum levels of ferritin and TIBC



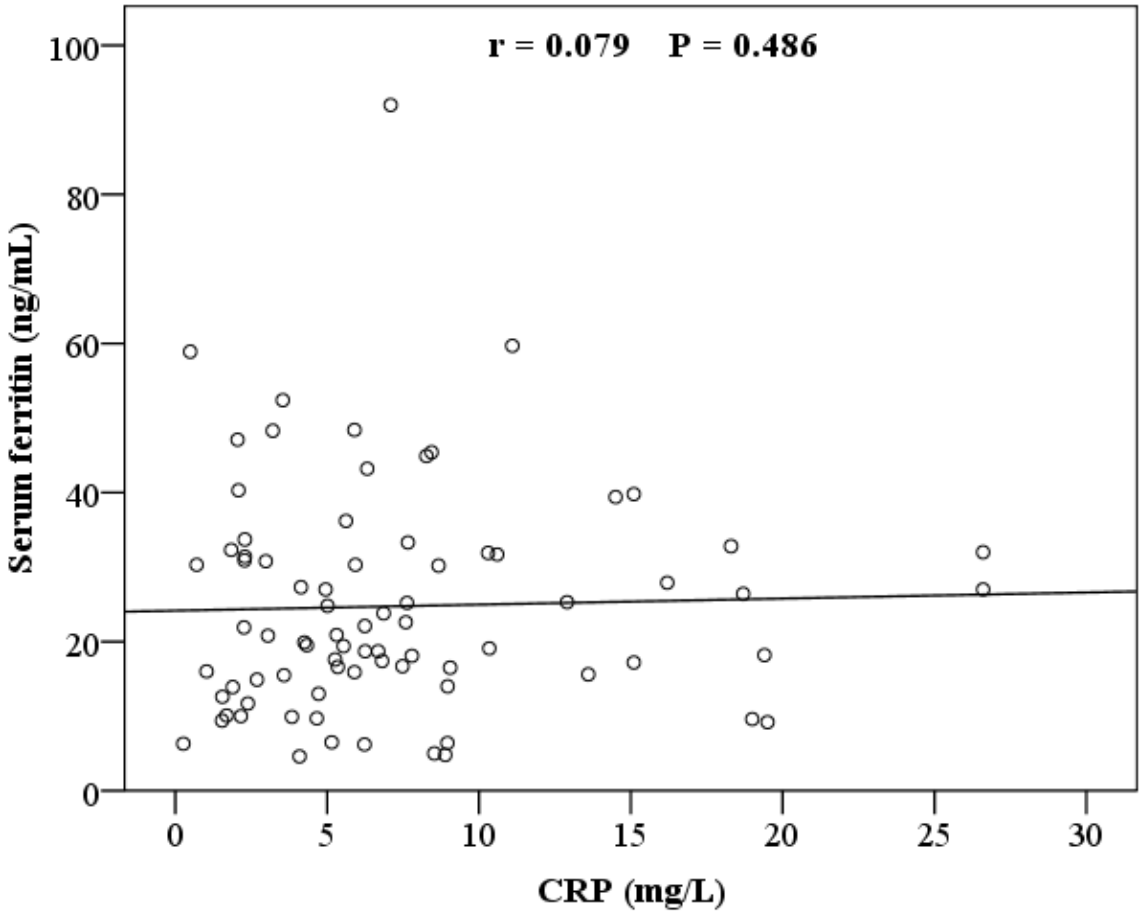
Serum ferritin levels bore a significant negative correlation with serum TIBC values.

Figure 11: Correlation analysis of serum ferritin and transferrin saturation



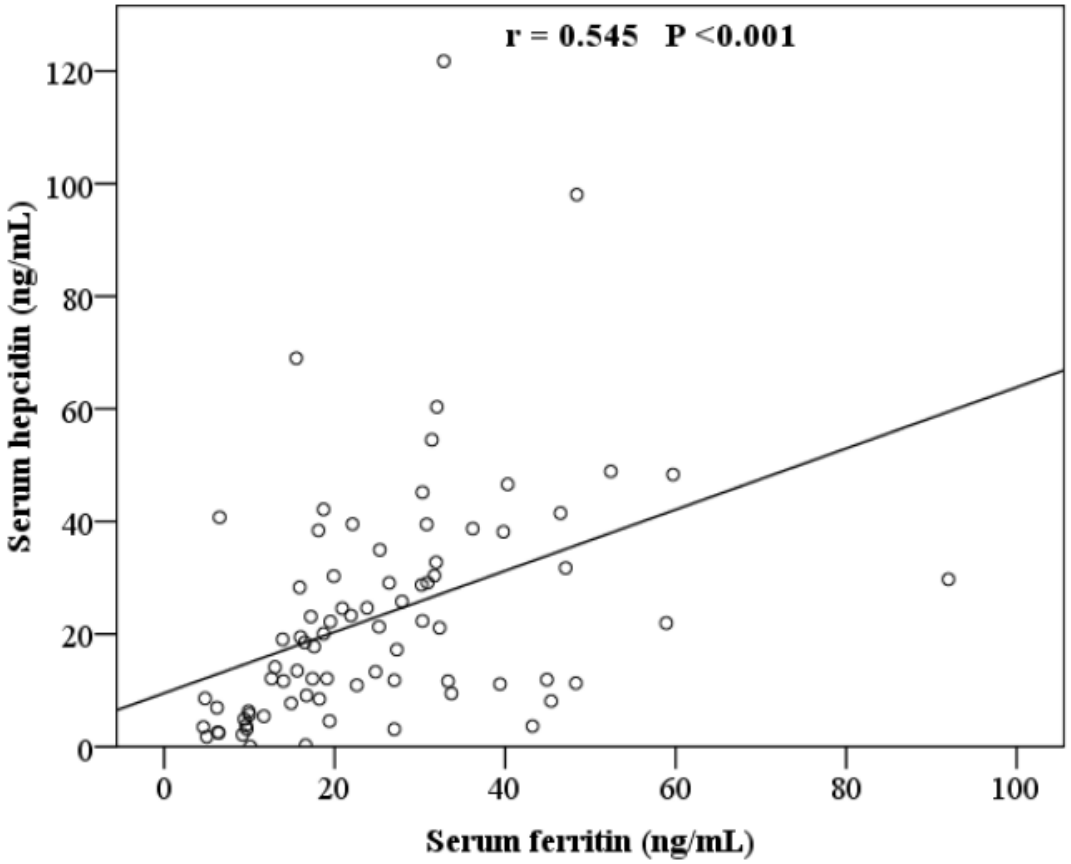
There tended to be a positive correlation between serum ferritin and transferrin saturation ($p = 0.056$).

Figure 12: Correlation analysis of serum levels of ferritin and CRP



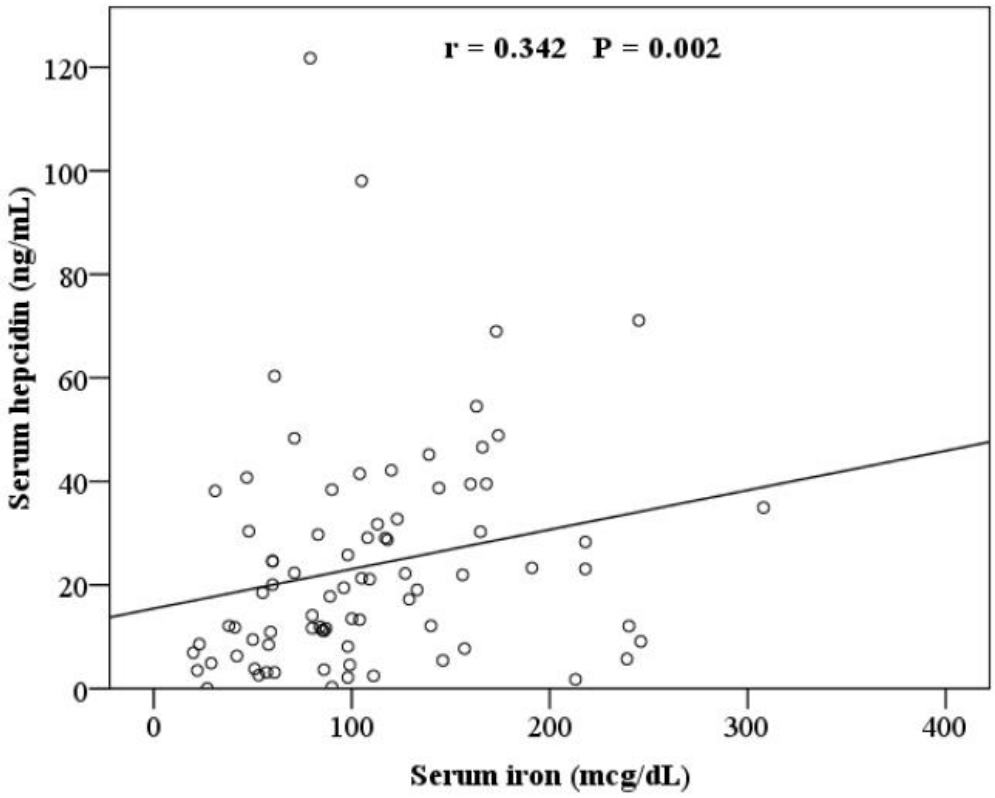
There was no significant correlation between serum levels of ferritin and CRP.

Figure 13: Correlation analysis of serum levels of hepcidin and ferritin



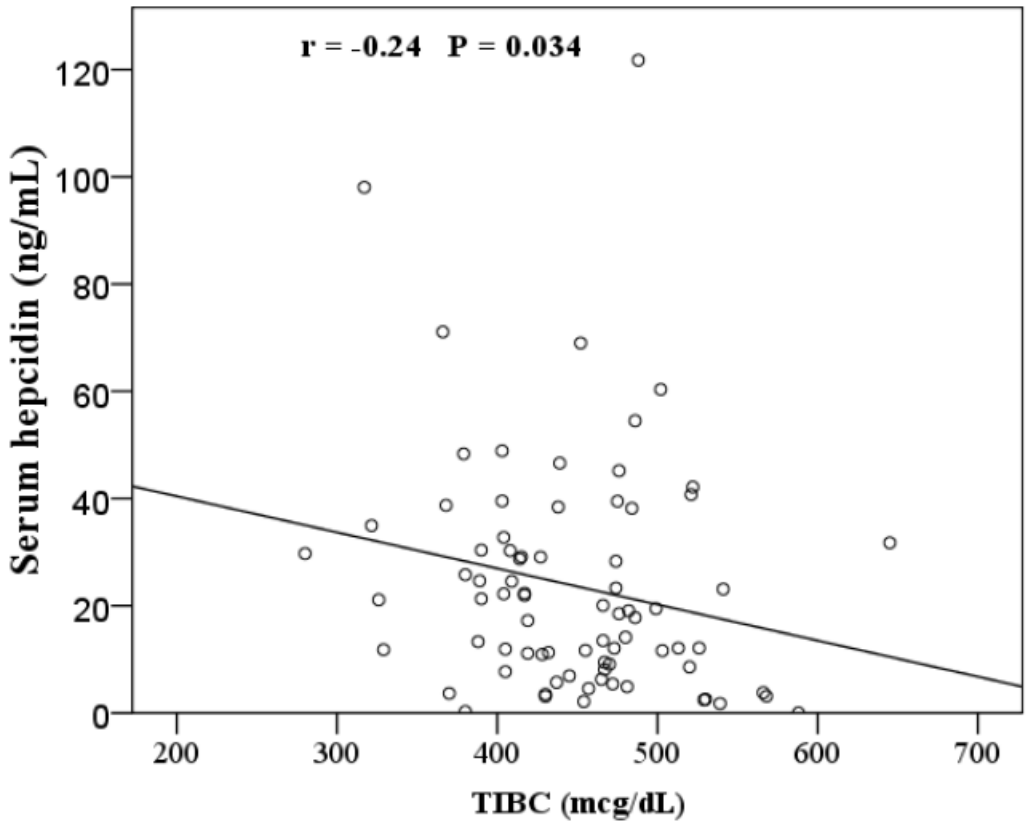
Serum hepcidin levels bore a significant positive correlation with serum ferritin levels.

Figure 14: Correlation analysis of serum levels of hepcidin and iron



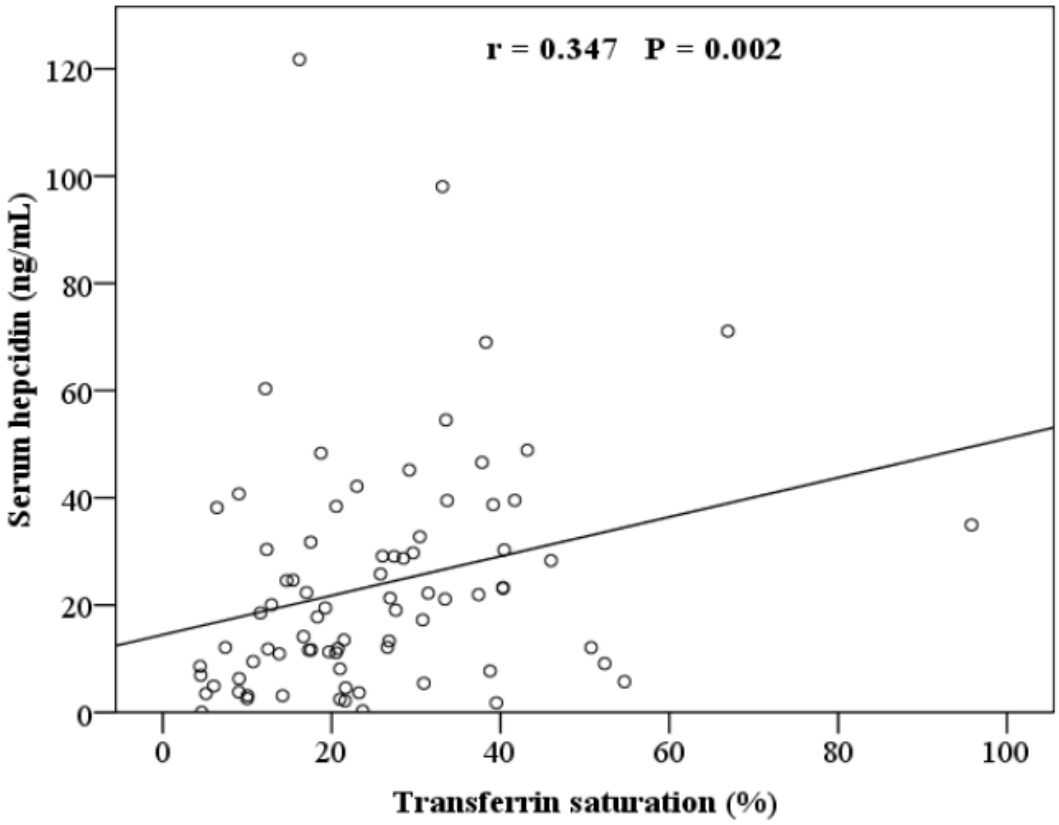
Serum hepcidin levels bore a significant positive correlation with serum iron levels.

Figure 15: Correlation analysis of serum levels of hepcidin and TIBC



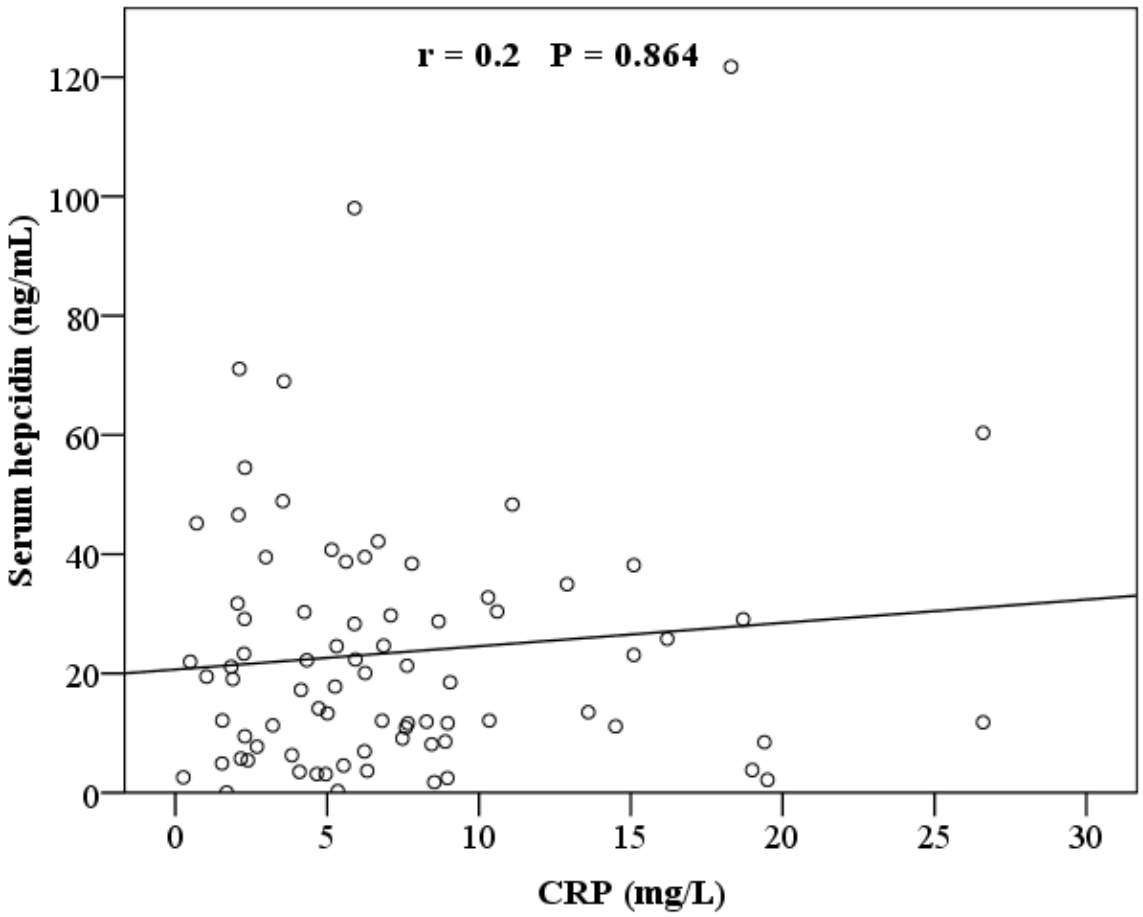
Serum hepcidin levels bore a significant negative correlation with values for TIBC.

Figure 16: Correlation analysis of serum levels of hepcidin and transferrin saturation



Serum hepcidin levels bore a significant positive correlation with transferrin saturation levels.

Figure 17: Correlation analysis of serum levels of hepcidin and CRP



There was no significant correlation between serum levels of hepcidin and CRP.

Table 5: Outcomes of pregnancies of the subjects

	Controls (mean ± SD or median [IQR])	**GDM (mean ± SD or median [IQR])**	**P value**
Gestational ages at delivery (days)	252.64 ± 13.4	258.13 ± 13.1	0.101
Birth weight of baby (kg)	2.81 [2.6 - 3.2]	2.66 [2.49 - 2.96]	0.088
APGAR score (out of 15)	14 [13 - 15]	13 [12.5 - 15]	0.535
Length of the baby (cm)	53 [52 - 56]	54 [52 - 57]	0.389

Data were analysed by Mann-Whitney U test or Student's t test, as appropriate.

The gestational ages at which delivery occurred, birth weights of the baby, the APGAR scores and lengths of the new-born babies were similar in both groups.

Table 6: Mode of delivery

Group	Mode of delivery			
	Normal vaginal delivery	**Forceps**	**Suction cup**	**LSCS**
Controls (N= 57)	29 (50.87%)	3 (5.26%)	4 (7.01%)	21 (36.84%)
GDM (N= 23)	10 (43.47%)	2 (8.69%)	2 (8.69%)	9 (39.13%)

There was no significant difference between the 2 groups, with regard to modes of delivery.

SUMMARY OF FINDINGS

1. Primigravidae, with and without GDM, did not differ with regard to values of haematocrit, BMI, serum ferritin, hepcidin, iron, TIBC, transferrin saturation and CRP.

2. Serum ferritin bore a significant positive correlation with serum transferrin saturation, and a significant negative correlation with TIBC values.

3. Serum hepcidin level bore significant positive correlation with serum iron, ferritin and transferrin saturation and a significant negative correlation with TIBC values.

3. The gestational ages at delivery, birth weight of the baby, the APGAR score, and length of the new-borns were similar in both groups.

4. There were no significant difference between the 2 groups, with regard to presence of a family history of diabetes mellitus, duration of iron supplementation and modes of delivery.

DISCUSSION

In pregnancy, serum ferritin levels have been shown to be lower compared to healthy non-pregnant women, reflecting a state of iron deficiency (Adediran et al., 2011; Alper et al., 2000). It is, therefore, expected that hepcidin expression would be downregulated during pregnancy to meet the additional needs of the growing fetus. Serum ferritin and hepcidin levels have been shown to decline in pregnancy, with levels being lowest in the third trimester (Adediran et al., 2011; Alper et al., 2000; Dao et al., 2013; Finkenstedt et al., 2012; Gyarmati et al., 2011; van Santen et al., 2013). Maternal serum ferritin levels have been shown to peak in the second trimester and decline thereafter (Larsson et al., 2008; Xiao et al., 2002).

Studies have shown that maternal serum hepcidin levels are significantly lower than fetal cord blood hepcidin levels (Rehu et al., 2010; van Santen et al., 2013). However, maternal hepcidin levels have been shown to negatively correlate with serum iron and transferrin saturation in cord blood, suggesting increased transfer of iron to the fetus (Dao et al., 2013; Koenig et al., 2014).Koenig et al. (2014) published a meta-analysis of studies on hepcidin in pregnancy, reviewing 10 human and 6 animal studies. Among the human studies, 4 were longitudinal and 6 were cross-sectional. These studies varied in their sample sizes, the time points (during pregnancy, at delivery or postpartum) at which samples were collected, the subjects studied (healthy pregnant subjects, with placental malarial infection, preeclamptic subjects etc) and type of samples (serum, plasma, cord blood or urine) used for estimation of hepcidin. Estimations of serum hepcidin in these studies were done by different methods such as enzyme-linked immune-sorbent assay (ELISA), mass spectrometry and cation-

exchange chromatography. It is difficult to make direct comparison of serum levels of hepcidin levels across these studies, as measurements were carried usingdifferent methods of assay. There is no established reference interval for serum hepcidin across the trimesters of pregnancy. The small number of studies in this area, variations in methods of estimation and lack of an established reference interval make it difficult to directly compare the results of above studies (Koenig et al., 2014).

Studies have shown that serum ferritin and hepcidin levels were elevated in patients with type 2 diabetes mellitus (Altamura et al., 2017; Andrews et al., 2015; Martinelli et al., 2012). Increased serum ferritin levels in pregnant women have been reported to be a significant risk factor for gestational diabetes mellitus (Amiri et al., 2013; Chen et al., 2006; Javadian et al., 2014; Kataria et al., 2018; Lao et al., 2001). The study by Derbent et al. (2013), carried out in a Turkish population, reported significantly elevated serum ferritin, hepcidin and iron levels in women with GDM, compared to women with impaired glucose tolerance (IGT) and pregnant women with normal glucose tolerance.

In the present study, serum levels of ferritin, hepcidin, iron, TIBC values and percentage of transferrin saturation, were similar in women with and without GDM. The above findings are in contrast to those of Derbent et al (2013). There are some similarities and a number of important differences between the findings and methodologies in the 2 studies. These are summarized in the tables below.

Similarities between the present study and that by Derbent et al (2013)

Feature	***Present study***	***Study by Derbent et al (2013)***
Gestational agewhenblood was taken for estimations of parameters of interest	24-28 weeks	24-28 weeks
Haematocrit values	Similar in those with and without GDM.	Similar in those with and without GDM.

Differences between the present study and that by Derbent et al (2013)

Feature	***Present study***	***Study by Derbent et al (2013)***
Sample size	82 in total (23 with GDM and 59 without GDM)	149 in total (30 with GDM, 72 without GDM and 47 with impaired glucose tolerance)
Gravida of subjects in the study	Subjects with and without GDM were all primigravidae.	Women with GDM were multipararous, while those without GDM were primigravidae.

Feature	***Present study***	***Study by Derbent et al (2013)***
Ages of subjects in the study	Ages of women with and without GDM were similar	Women with GDM were significantly older than women without GDM
BMI of subjects in the study	BMI and mid-arm circumference were found to be similar in both groups of women.	Women with GDM had significantly higher BMI values than women without GDM.
Iron and folic acid supplements	All subjects were on iron and folic acid supplements	None were on iron or folic acid supplements
Blood sample used for measurement of serum levels of ferritin, hepcidin and marker of iron stores	Fasting blood sample	Blood samples collected were in the non-fasting state.
Assay and instrument used for estimation of serum ferritin levels	Chemiluminescence assay, Siemens, ADVIA, Centaur System, XPi, UK	Electro-chemiluminescence assay, E170, Roche, Germany
ELISA kit used for estimation of serum hepcidn	Peninsula laboratories, USA	DRG Instruments, Marburg, Germany

Feature	***Present study***	***Study by Derbent et al (2013)***
Diagnosis of GDM made by	OGTT using 75 gm of oral glucose load	Glucose challenge test (GCT) (50 gm of glucose load), followed by anOGTT (100 gm of glucose load), for those who tested positive on the GCT.
Diagnostic criteria used for GDM	At least 1 abnormal value of plasma glucose for fasting, 1 hour or 2 hours samples, after a 75 gm glucose load, as per criteria of the International Association of the Diabetes and Pregnancy Study Groups (IADPSG)(ADA, 2016).	At least 2 abnormal values of plasma glucose for fasting, 1 hour, 2 hours and 3 hours samples, after a 100 gm glucose load (modified National Diabetes Data Group [NDDG] criteria) (Carpenter and Coustan, 1982)
Serum ferritin levels	Serum ferritin levels were similar in those with GDM and without GDM.	Serum ferritin levels were significantly higher in those with GDM than in

		those without GDM.
Feature	***Present study***	***Study by Derbent et al (2013)***
Serum hepcidin levels	Serum hepcidin levels were similar in those with GDM and without GDM.	Serum hepcidin levels were significantly higher in those with GDM than in those without GDM.
Serum iron levels	Serum iron levels were similar in those with GDM and without GDM.	Serum iron levels were significantly higher in those with GDM than in those without GDM.
Serum CRP levels	Serum CRP levels were similar in those with and without GDM.	Serum CRP levels were significantly higher in those with GDM than in those without GDM.

The above table clearly shows that the subjects and methodologies in the present study and that by Derbent et al (2013) are different in numerous ways. This may account for many of the differences seen in the results of the 2 studies. The differences in the methodologies used in the 2 studies, thus, make it difficult to make direct comparisons of the data obtained in them.

The present study shows that serum ferritin levels were similar in women with and without GDM. It has been reported that increased levels of serum ferritin in pregnant women were associated with increased risk of gestational diabetes mellitus (Chen et al., 2006; Kataria et al., 2018). However, the present study does not show this association. This difference could be due to the fact that in those earlier studies multiparous women were included in the GDM group, different glucose loads were used for the GTT and the BMI were significantly higher in women with GDM.

Maternal serum ferritin levels showed positive correlation with percentage of transferrin saturation and a significant negative correlation with values for TIBC. These findings are in agreement with other studies that showed that maternal serum ferritin levels correlated significantly with percentage of transferrin saturation and TIBC (Amiri et al., 2013; Kataria et al., 2018; Raza et al., 2011).

In the present study, maternal serum hepcidin levels showed a significant positive correlation with serum levels of ferritin, iron, percentage of transferrin saturation and a significant negative correlation with TIBC. These findings are in agreement with other studies that showed that maternal hepcidin levels correlated significantly with markers of maternal iron status (Finkenstedt et al., 2012; Rehu et al., 2010; van Santen et al., 2013).

In the present study, body mass index (BMI) was not significantly different between women with and without GDM. However, the calculation of BMI in pregnancy is generally not held to be reliable (Nuttall, 2015; Widen and Gallagher, 2014). Hence, the mid-arm circumference (MAC) of each subject was also used in the present study.

Studies have been used MAC as an indicator of obesity (Fakier et al., 2017; Okereke et al., 2013). The MAC of the subjects in 2 groups in the present study wassimilar. MAC hasalso been reported to be similar in pregnant women, irrespective of their taking iron supplements(Hedengran et al., 2016). This parameter also did not differ between pregnant women with favourable and favourableoutcomesof pregnancy (van Santen et al., 2013; Schulze et al., 2008).

Iron-related parameters in blood are known to be affected by iron supplements (Alizadeh and Salehi, 2016; van Santen et al., 2014). Hence, these parameters in the present study should have been estimated under supplement-free conditions. However, it is mandatory to supplement iron and folic acid in pregnant women, as per national and international guidelines (Kumar, 1999; Peña-Rosas et al., 2012). It would have been unethical to have withheld iron and folic acid supplements from the pregnant women in the present study. Therefore, women in both control and GDM groups received iron and folic acid supplements. It is hoped that intake of iron in both groups would have controlled for the possible confounding effects of iron on the parameters of interest, namely, serum levels of ferritin, hepcidin, iron, transferrin saturation percentage and TIBC values. No significant differences were observed in values of these parameters between the 2 groups. Serum hepcidin levels have been shown to increase in response to oral iron (Moretti et al., 2015). However, interestingly, it has been reported in a recent study that serum hepcidin levels were lower in pregnant women who took adequate iron supplements (Friedrisch and Friedrisch, 2017). The exact basis of this observation is not clear.

Pregnancy is a pro-inflammatory state. Pregnant women have been shown to have elevations in inflammatory markers, compared to healthy non-pregnant women (Nemeth et al., 2003). In the study by Derbent et al (2013), serum C-reactive protein (CRP) levels were significantly higher in women with GDM, than women without GDM. However, the present study has shown that serum CRP levels were similar in 2 groups. Factors that may contribute to this difference include the facts that women with GDM in the study by Derbent et al (2013) were older than the control subjects, were multiparous (while those in the control group wereprimigravidae) and had higher values for BMI than control subjects.

In present study, the various parameters related to outcomes of pregnancy, such as gestational age at delivery, birth weight of the newborns, the APGAR score and the length of the newborns were similar in 2 groups. Otherstudies have shown that there were significant adverse outcomes of pregnancies in women with GDM (Boriboonhirunsarn et al., 2006; Mayor, 2017; Wendland et al., 2012). The women with GDM in the above studies were older than the subjects in the present study and were also obese. These factors may have influenced the outcomes of their pregnancies. The data for the women who did not deliver in CHAD is not as reliable as that obtained from the medical records in CHAD, as the former were obtained by phone interviews with the women some weeks/months after they had delivered. Hence, it is not possible to draw definitive conclusions from these observations.

CONCLUSION

There were no significant differences in levels of serum ferritin and other iron-related parameters in women with and without GDM. Hence, based on the data in this study, it does not appear that increased serum ferritin levels may be useful as a marker for development of GDM.

LIMITATIONS OF THE STUDY

1. One of the secondary objectives of the study was to follow up the subjects to determine outcomes of pregnancies. However, some of them were yet to deliver at the time of submission of this thesis. Hence, it has not been possible to document all these outcomes to complete the comparisons between the 2 groups.

2. It was not possible to estimate additional parameters linked to diabetes mellitus and body iron status (such as serum insulin, complete blood counts, and serum levels of soluble transferrin receptor), due to financial constraints. These parameters may have provided additional useful information.

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APPENDIX 1: IRB APPROVAL LETTER

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

February 23, 2017

Dr M Gopinath,
PG Registrar,
Department of Biochemistry,
Christian Medical College,
Vellore - 632 004.

Sub: **Fluid Research Grant NEW PROPOSAL:**
Serum Ferritin In Gestational Diabetes Mellitus
Dr. M. Gopinath, Employment number:21330, PG Registrar, Dr. Molly Jacob, Employment number: 14509, Professor and Head, Dr. Joe Varghese, Employment no: 20405, Professor, Dr. Jasmine Prasad, Employment no: 20080, Professor and Head, Dr. Arthi T S, Employment no: 33380, Senior Resident, Department of Biochemistry.
Ms Thenmozhi M, Employment no: 32347, Associate research officer, Department of Biostatistics.

Ref: IRB Min No: 10423 [OBSERVE] dated 05.12.2016

Dear Dr Gopinath,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Serum ferritin in gestational diabetes mellitus " on December 05th 2016.

The Committee reviewed the following documents:

1. IRB Application format
2. Proforma
3. Information Sheet and Consent Form (English, Tamil, Hindi)
4. Signature Pages
5. No. of documents 1 - 4

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on December 05th 2016 in the BRTC Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, Research), Additional Vice Principal , Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC,Vellore	Internal, Clinician
Dr. B. J. Prashantham	MA(Counseling Psychology), MA (Theology), Dr. Min (Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Dr. Ratna Prabha	MBBS, MD (Pharma)	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist
Dr. Rekha Pai	BSc, MSc, PhD	Associate Professor, Pathology, CMC, Vellore	Internal,Basic Medical Scientist
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Simon Pavamani	MBBS, MD	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. Rajesh Kannangai	MD, PhD.	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Ms. Grace Rebekha	M.Sc., (Biostatistics)	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. Anuradha Rose	MBBS, MD, MHSC (Bioethics)	Associate Professor, Community Health, CMC, Vellore	Internal, Clinician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
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Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Santhanam Sridhar	MBBS, DCH, DNB	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "Serum ferritin in gestational diabetes mellitus" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

Fluid Grant Allocation:

A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment.

Yours sincerely,

Dr. Biju George
Secretary(Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min No: 10423 [OBSERVE] dated 05.12.2016

APPENDIX 2: INFORMED CONSENT AND INFORMATION SHEET

Study number:

Department of Biochemistry & Department of Community Health
Christian Medical College, Vellore – 632002

Informed consent form for subjects

Study title: Serum ferritin in gestational diabetes mellitus

Subject name:

Hospital number:

Date of birth:

I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask Dr.Gopinath any questions I had. [ ]

I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

I understand that if I agree to participate in the study, a blood sample of 10ml will be collected from me. [ ]

I understand that this will not affect my health in a foreseeable way. [ ]

I understand that the blood sample taken will be used only for research purposes. If there is any sample remaining after this study is completed, I give permission for the sample to be stored and used for related studiesin the future. [ ]

I understand that my identity will not be revealed in any information released to third parties or published and all my medical information will be kept confidential.[ ]

I agree to take part in the above study of my own free will.[ ]

Subject's name and signature/thumb impression (with date)		

Witness's name and signature (with date):

Investigator name and signature (with date):

SERUM FERRITIN IN GESTATIONAL DIABETES MELLITUS

INFORMATION SHEET FOR STUDY PARTICIPANTS

The Department of Biochemistry at Christian Medical College, Vellore, in association with the Department of Community Health is carrying out a study to look for changes in blood levels of ferritin in pregnant women who develop diabetes mellitus during their pregnancy. Ferritin is a substance in blood that reflects iron stores in the body. High levels of iron in the body may be associated with increased risk of developing diabetes during pregnancy. This study is being done to help doctors to understand better the relationship between iron levels in the body and diabetes mellitus that develops during pregnancy. For this study, we require 10 ml of blood. We would like to ask if you are willing to provide 10ml of blood for this purpose.

The blood sample collected will be used only for research purposes. If there is any sample left over after this study is completed, we would like to request you for permission to store it and use it for related research studies in the future. Collection of blood will not cause harm to your health and will be done at the same time that blood is taken for your routine tests. The medical information you give us will be kept confidential.

You may not benefit directly by participation in the study. However, if you are willing to provide the blood sample, it will help us understand how ferritin levels may vary in diabetes mellitus during pregnancy and may help, in the future, to improve detection and treatment for this condition.

If you are not willing to participate in the study, you are free to do so. It will not affect the treatment you will receive from the hospital. Participation in the study does not entitle you to concession or any other special treatment. If you have further queries, please contact us, using one of the numbers given below.

Dr. Gopinath M,
PG Registrar,
Department of Biochemistry,
Christian Medical College,
Vellore – 632002
Contact number: 978 643 7148

Dr. Jasmine Prasad,
Professor,
Department of Community Health,
Christian Medical College,
Vellore – 632002
Contact number: 0416 - 2284207

Dr. Molly Jacob,
Professor & Head,
Department of Biochemistry,
Christian Medical College,
Vellore – 632002
Contact number: 0416 - 2284267

Study number:

உயிர் வேதியியல் துறைமற்றும்சமூக நலத்துறை
கிறித்துவ மருத்துவக்கல்லூரி ,வேலூர்.
விபரம்அறிந்துதெரிவிக்கும்இசைவு

ஆய்வுதலைப்பு:கர்ப்பகாலநீரிழிவுநோய்சீரம்பெற்றிடின்நிலைகள்

ஆய்வில்பங்கேற்பவரின்பெயர்:

மருத்துவமனைஅடையாளஎண்:

பிறந்தநாள்/வயது (வருடங்களில்): _____/_____/_____

திருமதி _____ஆகியநான்

இந்தஆய்வின்தகவல்படிவத்தைபடித்தும்,
அதன்விவரங்களைமரு.கோபிநாத்முலம்கேட்டும்புரிந்துகொண்டேன்( ).

இந்தஆராய்ச்சிக்காக 10
மில்லிஇரத்தம்கொடுக்கமுழுமனதுடன்சம்மதிக்கிறேன்.இதனால்உடல்நலத்திற்குஎந்தவித பாதிப்பும்ஏற்படாதுஎனஅறிந்துகொண்டேன். மேலும்,
இந்தஆராய்ச்சிக்குபயன்படுத்தியபின்மீதமாகும்இரத்தத்தைசேகரித்துபின்சம்பந்தப்பட்டஆராய்ச்சிக்குபயன்படுத்தவும்சம்மதிக்கிறேன்( ).

இந்தஆய்வில்கலந்துகொள்ளஎன்னையாரும்கட்டாயப்படுத்தவில்லை.எனதுசுயவிருப்பத்துடன்இந்தஆய்வில்பங்கேற்கசம்மதிக்கிறேன்.மேலும்,
இந்தஆய்விலிருந்துஎந்நேரமும்வெளிவரஎனக்குஅனுமதியுள்ளது.
இதனால்எனக்குஅளிக்கப்படும்சிகிச்சையில்எந்தவிதவேறுபடும்இருக்காதுஎனஎனதுமருத்துவர்உறுதியளித்துள்ளார் ( ).

எனதுமருத்துவவிபரங்கள்ஆய்வில்உபயோகிக்கஒப்புக்கொள்கிறேன்.இருப்பினும்எனதுதனிப்பட்டஅடையாளம்/ தகவல்,
மற்றவரிடமோ/மருத்துவஇதழிலோவெளியிடப்படமாட்டாதுஎன்பதையும்தெரிந்துகொண்டேன்( ).

இந்தஆய்வின்முடிவு/தகவலைஅறிவியல்நோக்கத்திற்குமட்டும்பயன்படுத்தநான்சம்மதிக்கிறேன்( ).

இந்தஆய்வில்பங்கேற்கமுழுமனதுடன்சம்மதிக்கிறேன்( ).

ஆய்வில்பங்கேற்பவரின்கையொப்பம்/கைரேகை: (தேதி)		

சாட்சியின்பெயர்/ கையொப்பம்/கைரேகை (தேதி:);

ஆராய்ச்சியாளர்கையொப்பம்(தேதி):

உயிர் வேதியியல் துறைமற்றும்சமூக நலத்துறை,
கிறித்துவமருத்துவக்கல்லூரி , வேலூர்-

கர்பகால நீரிழிவு நோய் சீரம் பெற்றிடின்நிலைகள்
ஆய்வில் பங்குபெருவோருக்கான தகவல் தாள்

உயிர் வேதியியல் துறை , சமூக நலத்துறையுடன் இணைந்து கர்பகாலத்தில் ஒரு சிலருக்கு ஏற்படும் நீரிழிவு நோயின் பொழுது , இரத்தத்தில் உள்ள பெற்றிடின் அளவின் மாற்றத்தை பற்றி கண்டறிய ஆய்வு மேற்கொள்ளஉள்ளோம் . பெற்றிடின், இரத்தத்தில் உள்ள இரும்புச்சத்தின் அளவை சரிசமமாக வைத்துக்கொள்ளும்.உடலில் இரும்புச்சத்து அதிகமாக இருந்தால் கர்பகாலத்தில் நீரிழிவு நோய் வர வாய்ப்புள்ளது. இந்த ஆராய்ச்சியின் கர்பகால நீரிழிவு நோயில் இரும்புசத்து எவ்வாறு கையாளப்படுகிறது என்பதை அறிய உதவும்.

இவ்வராய்ச்சிக்காக 10மில்லி இரத்தம் தேவை படுகிறது.

தாங்கள் தங்கள் சுயவிருப்பத்துடன் இவ்வாராய்ச்சியில் பங்கு பெற 10மில்லி அளவு இரத்தம் தரவேண்டியிருக்கும். இதனால் தங்கள் உடலுக்கு எவ்வித தீங்கும் ஏற்படாது.ஆராய்ச்சிக்கான இந்த இரத்தத்தை , தாங்கள் இதர இரத்தபரிசோதனைக்கு வரும்பொழுது , தங்கள் அனுமதியுடன் தங்கள் முன்னிலையில்பெற்றுக்கொள்ளப்படும்.மீதமாகும் இரத்தத்தை சேகரிக்கவும், அதை பின் ஆராய்ச்சிக்காக பயன்படுத்தவும் தங்களின் அனுமதி கோருகிறோம்.
தங்களது இரத்தம் ஆராய்ச்சிக்காக மட்டுமே உபயோகிக்கப்படும்.
ஆராய்ச்சிக்காக அறியப்படும் தகவல்கள் நம்பகமான முறையில் பாதுகாக்கப்படும்.
இவ்வாராய்ச்சியில் பங்கு பெறுவதால் தாங்கள் நேரடியாக பயன்பெற இயலாது. ஆனால் இதன் மூலம் அறியப்படும் தகவல்கள் பிற்காலத்தில் இந்நோயை சரியாக கையாள உதவும்.

தங்களுக்கு இந்த ஆராய்ச்சியில் பங்குபெற விருப்பமில்லை என்றால் தயங்காமல் கூறலாம். அதனால், இந்த மருத்துவமனையில் தங்களுக்கு தரப்படும் சேவை பாதிக்கப்படாது.
மேலும் விபரங்கள் அறிய மற்றும் சந்தேகங்களை தெரியப்படுத்த கீழ்காணும் நபர்களை அவர்கள் தொலைபேசி எண்களுக்கு தொடர்புகொள்ளவும்:

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மரு. மோலி ஜேக்கப்,
உயிர் வேதியியல் துறை,
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வேலூர்– 632002
தொலைபேசி எண்: 0416- 2284267

APPENDIX 3: PARTICIPANT'S PROFORMA

GDM/ Control/ Study number:

DEPARTMENT OF BIOCHEMISTRY AND DEPARTMENT OF COMMUNITY HEALTH
CHRISTIAN MEDICAL COLLEGE, VELLORE – 632002
SERUM FERRITIN IN GESTATIONAL DIABETES MELLITUS

PROFORMA FOR DETAILS OF PARTICIPANT

NAME	AGE	HOSPITAL NO	SPOUSE NAME	PHONE NO

LMP	EDD	IFA started from (weeks)	GA at the time of OGTT

Family history of diabetes mellitus			Any complications of pregnancy
Mother	Father	Both	
H/o drug for any illness			H/o blood transfusion

Hb/Hct	Blood grp	Height	Weight	MAC	BP

OGTT	Fasting	1h post glucose	2h post glucose
Plasma glucose (mg/dL)			

CRP (mg/L)	Serum ferritin (ng/mL)	Serum iron (µg/dL)	TIBC (µg/dL)	Transferrin saturation%	Serum hepcidin (ng/mL)

Gest Age at delivery	BP at time of admission for delivery	Mode of delivery	Any pregnancy related complication that developed

Sex of baby		Birth weight	Length of baby	APGAR score
Male	Female			

APPENDIX 4: MASTER DATA SHEET

Category	Fasting plasma glucose (mg/dL)	One hr post OGTT plasma glucose (mg/dL)	Two hr post OGTT plasma glucose (mg/dL)	Age (years)	Gestational age at recruitment (days)	Hct (%)	MAC (cm)	Duration for which iron was prescribed (days)	Ferritin (ng/mL)	Iron (µg/dL)	UIBC (µg/dL)	TIBC (µg/dL)	Transferrin saturation (%)	CRP (mg/L)	Hepcidin (ng/mL)
Control	74	112	107	23	196	35.5	28	157	33.3	87	416	503	17	8	11.63
Control	76	112	118	26	179	34.8	26	0	30.3	71	346	417	17	6	22.33
Control	81	144	116	18	183	35	-	104	20.8	122	317	439	28	3	-
Control	74	147	142	21	162	38.5	29	50	15.6	100	366	466	21	14	13.49
Control	81	136	115	23	162	33.7	22.5	78	6.3	53	477	530	10	0	2.57
Control	80	99	72	21	162	37.5	23	78	13.9	133	349	482	28	2	19.07
Control	81	151	121	24	169	33	24.5	85	12.6	140	386	526	27	2	12.11
Control	78	173	126	22	162	36	23.5	64	6.4	111	418	529	21	9	2.45
Control	82	173	145	29	162	33.8	25	78	30.2	118	296	414	29	9	28.73
Control	76	130	97	25	162	37	23	15	-	-	-	-	-	-	-
Control	80	129	78	28	148	33	23	120	16	96	403	499	19	1	19.46
Control	96	108	119	21	148	37	29	127	26.4	117	310	427	27	19	29.08
Control	95	175	148	33	162	36.2	29	78	31.9	123	281	404	30	10	32.76
Control	82	130	120	18	180	34.9	28	152	18.7	60	406	466	13	6	20.06
Control	80	139	111	21	169	37	22	141	59.7	71	308	379	19	11	48.34
Control	82	118	127	21	176	36	28	141	16.5	55	421	476	12	9	18.51

Control	76	142	124	18	148	34.5	20	120	10.1	27	561	588	5	2	0.01
Control	82	134	131	24	162	34.5	26	134	11.7	146	326	472	31	2	5.43
Control	81	120	110	23	148	38.5	37	8	39.4	86	333	419	21	15	11.13
Control	86	152	124	27	218	33.6	22	134	16.6	90	290	380	24	5	0.28
Control	90	195	135	20	148	36.8	23	36	13	80	400	480	17	5	14.12
Control	88	146	93	21	169	37.2	32	57	22.1	168	235	403	42	6	39.53
Control	81	169	134	20	162	36.2	25	99	17.6	89	397	486	18	5	17.79
Control	83	117	109	21	176	37.9	23	92	-	245	121	366	67	2	71.08
Control	84	118	149	22	190	34.6	24	64	46.5	104	-	-	-	-	41.48
Control	89	134	113	24	190	34.3	26	106	23.8	60	329	389	15	7	24.65
Control	86	174	117	23	268	36	23	219	52.4	174	229	403	43	4	48.9
Control	73	161	147	23	200	36.59	22.5	102	10	239	198	437	55	2	5.72
Control	87	107	134	21	175	35.5	23.5	98	14.9	157	248	405	39	3	7.71
Control	81	163	141	22	214	35.5	26	95	24.8	104	284	388	27	5	13.3
Control	77	115	114	18	176	36.1	26	99	30.9	108	307	415	26	2	29.13
Control	86	176	116	21	195	44	29	132	33.7	50	417	467	11	2	9.46
Control	80	176	134	25	173	40.7	27.5	71	9.7	57	511	568	10	5	3.13
Control	83	134	122	24	182	34.4	26	143	20.9	60	349	409	15	5	24.56
Control	81	125	119	29	155	35.5	25	80	40.3	166	273	439	38	2	46.6
Control	79	138	103	26	182	36.5	23	100	45.4	98	369	467	21	8	8.1

Control	79	120	99	21	182	45.1	26.5	98	43.2	86	284	370	23	6	3.66
Control	82	113	118	22	183	36	24	134	27	61	369	430	14	5	3.11
Control	86	165	114	24	192	37.9	23.5	126	6.5	47	474	521	9	5	40.73
Control	88	126	141	18	179	35.2	25.5	83	48.4	105	212	317	33	6	98.04
Control	88	145	135	24	171	36.4	27.5	117	32	61	441	502	12	27	60.34
Control	85	167	134	25	192	35	26	83	18.7	120	402	522	23	7	42.14
Control	83	126	114	28	205	34.8	29.5	46	19.9	165	243	408	40	4	30.29
Control	88	128	121	19	190	36.5	25	113	6.2	20	425	445	4	6	6.92
Control	86	149	111	32	169	34.2	34.2	49	25.2	105	285	390	27	8	21.3
Control	80	125	123	21	166	33.7	20	107	19.4	99	358	457	22	6	4.55
Control	80	124	117	20	187	34.2	25.5	83	48.3	85	347	432	20	3	11.28
Control	74	108	79	22	196	38.4	22.5	72	27.3	129	290	419	31	4	17.24
Control	78	104	108	23	194	35.5	26.5	118	27.9	98	282	380	26	16	25.8
Control	80	151	102	23	-	36	24	-	92	83	197	280	30	7	29.73
Control	82	101	97	22	229	34.3	23	135	31.4	163	323	486	34	2	54.51
Control	78	82	72	25	227	34	26.5	172	15.5	173	279	452	38	4	68.99
Control	83	104	77	18	185	35.3	21.5	39	27	41	288	329	12	27	11.8
Control	76	127	122	21	148	37.1	28	29	19.5	127	277	404	31	4	22.21
Control	80	137	107	22	142	39.2	27.5	98	21.9	191	283	474	40	2	23.31
Control	82	158	121	20	185	37.9	23.5	118	14	80	375	455	18	9	11.67

Control	90	161	128	24	199	36.6	22	120	17.4	240	233	473	51	7	12.08
Control	78	81	127	21	162	35.4	23	78	9.9	42	423	465	9	4	6.28
Control	77	114	105	23	162	33.2	31	78	31.7	48	342	390	12	11	30.39
GDM	71	215	166	21	184	34.9	22.5	100	58.9	156	261	417	37	0	21.95
GDM	78	200	167	19	183	35.7	-	43	9.6	51	515	566	9	19	3.79
GDM	93	82	101	20	165	33.3	26	109	9.4	29	452	481	6	2	4.91
GDM	93	217	193	20	173	38.5	24	145	39.8	31	453	484	6	15	38.18
GDM	92	163	127	30	190	35.9	26	162	18.1	90	348	438	21	8	38.41
GDM	100	165	117	22	114	35.3	26	86	4.6	22	408	430	5	4	3.5
GDM	92	140	124	24	168	37.6	28	28	9.2	98	356	454	22	20	2.15
GDM	93	126	132	23	198	33.1	28	114	4.8	23	497	520	4	9	8.57
GDM	106	178	156	29	170	36	27	16	17.2	218	323	541	40	15	23.1
GDM	98	128	129	19	173	38.2	29	75	36.2	144	224	368	39	6	38.73
GDM	97	170	108	18	196	36.7	25	84	25.3	308	13.5	321.5	96	13	34.96
GDM	92	143	127	19	193	35.9	31	88	19.1	38	475	513	7	10	12.1
GDM	96	176	144	25	210	38.2	28	70	5	213	326	539	40	9	1.78
GDM	95	171	169	20	185	35.9	28.5	104	15.9	218	256	474	46	6	28.31
GDM	96	149	131	30	176	36.7	24	71	30.3	139	337	476	29	1	45.18
GDM	102	236	182	22	165	38	31	66	32.8	79	409	488	16	18	121.75
GDM	80	260	234	21	118	36.5	26	57	30.8	160	315	475	34	3	39.49

GDM	93	137	112	24	239	35.3	21.5	171	47.1	113	532	645	18	2	31.75
GDM	83	178	169	23	144	42	23	55	32.3	109	217	326	33	2	21.11
GDM	102	210	184	24	174	33.8	26.5	90	16.7	246	224	470	52	7	9.09
GDM	95	140	173	29	162	36.7	24	27	44.9	84	321	405	21	8	11.91
GDM	90	186	153	24	164	36	25	66	22.6	59	369	428	14	8	10.92
GDM	81	185	134	28	215	35.7	24.5	110	18.2	58	-	-	-	19	8.46

Hct - Haematocrit

MAC - Mid-arm circumference